Modulation of human neutrophil apoptosis by tumour necrosis factor-α

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Abstract

Neutrophil apoptosis is an important process in the control of inflammation. This event results in a shut down of neutrophil secretory responses and initiates the phagocytic clearance of intact senescent cells by a novel recognition and uptake pathway that does not incite a pro-inflammatory macrophage response. While the mechanisms involved in regulating neutrophil survival and death are poorly understood, there is now considerable evidence to indicate that this process is not immutable since the rate at which these cells undergo apoptosis, at least *in vitro*, can be altered. Indeed, many pro-inflammatory mediators have been demonstrated to inhibit neutrophil apoptosis *in vitro*, suggesting that such agents act not only in a priming or secretagogue capacity but also increase neutrophil functional longevity by delaying apoptosis. We have examined whether this hypothesis holds true for all neutrophil priming agents, in particular TNFα, a potent neutrophil priming agent which has been variably reported to either induce, delay, or have no effect on the rate of constitutive neutrophil apoptosis.

We have shown that incubation of neutrophils with various priming and/or activating agents for 20 hr either inhibited (LPS, LTB_4, GM-CSF, IP6) or had no effect on (fMLP, PAF) the rate of neutrophil apoptosis. TNFα however, despite causing a significant inhibition of apoptosis at 20 hr, promoted apoptosis in a subpopulation of cells at earlier times (<12 hr) when the constitutive rate of apoptosis is still low; this

effect was not observed with any of the other priming or activating agents examined at this time.

The early pro-apoptotic effect of TNFα was confirmed by DNA fragmentation and propidium iodide binding and shown to be concentration-dependent with a near-identical EC_{50} value (2.8 ng/ml) to that observed for TNFα-priming of fMLP-stimulated superoxide anion generation. Moreover, the early cytocidal effect of this cytokine was detectable within 2 hr, abolished by TNFα neutralizing antibody, and was not associated with any change in cell viability or recovery. Of note, TNFα-stimulated apoptosis was abolished by pre-incubation of neutrophils with selective blocking antibodies to both the TNFR55 (which contains the classical death-domain sequence and is entirely responsible for the TNFα priming effect in suspension neutrophils) and TNFR75 receptor subtypes. Moreover, the TNFR55-selective mutants (E146K, R32W-S86T) induced neutrophil apoptosis but with a potency 14-fold lower than wild type TNFα, while the TNFR75-selective mutant (D143F) did not induce apoptosis. These data indicate that TNFα has the ability apparently unique to this priming agent to induce apoptosis in human neutrophils at early time points via a mechanism whereby the TNFR75 facilitates and permits TNFR55-mediated induction of cell death. Of relevance to the inflamed site, the ability of TNFα to accelerate apoptosis was completely lost if neutrophils were primed with PAF (1 μM, 10 min) or aged for 6 hr, prior to TNFα addition.

The pro-apoptotic effect of TNFα was not mimicked by cell-permeable synthetic ceramides or neutral sphingomyelinase, suggesting that a sphingomyelin/ceramide triggered death pathway as observed in certain leukaemic cell lines may not be the principal signalling route employed by TNFα in human neutrophils. Inhibition of p38 MAP kinase and MAPKK-1 potentiated the pro-apoptotic effect of TNFα, while suppressing the protective effect of LPS at a later (20 hr) time, implicating the p38 and p42/44 MAP kinases in the protection of neutrophils against apoptotic stimuli.

Finally, although human neutrophils appear quite resistant to oxidant-induced killing (e.g. by H_2O_2, hyperoxia or NADPH oxidase activation), and wortmannin which ablates superoxide anion generation in these cells actually augmented TNFα-induced killing, cell culture under hypoxic conditions was the one intervention that resulted in a dramatic abrogation of the pro-apoptotic effect of TNFα. This latter finding implicates either an oxygen-dependent step in TNFα-mediated neutrophil apoptosis or the capacity for the survival response triggered by anoxia to override the TNFα killing effect.

These data suggest that the major increase in TNFα which is such a characteristic feature of the early inflammatory response may provide a very important signal for neutrophil clearance via the induction of apoptosis. The unconventional dependency of this response on both TNFR55 and TNFR75 and the restriction of the latter receptor subtype to myeloid cells suggests a unique mode of action of TNFα in these cells and the potential for selective therapeutic intervention.

Abbreviations

ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BSA	Bovine serum albumin
C5a	Complement fragment 5a (anaphylatoxin)
Ca^{2+}	Calcium ion
$CaCl_2$	Calcium chloride
cAMP	cyclic adenosine monophosphate
CD	Cluster of differentiation (of leukocyte antigens)
CR	Complement receptor (types 1 and 3)
db-cAMP	dibutyryl cAMP
DMSO	Dimethyl sulphoxide
DNA	Deoxy-ribonucleic acid
DR3	Death domain containing receptor 3
ELISA	Enzyme linked Immunosorbant assay
ERK	extracellular signal-related kinase
FADD	Fas-associated protein with death domain
FcR	Receptor for Fc-piece of immunoglobulin
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1β converting enzyme
fMLP	*N*-formyl-methionyl-leucyl-phenylalanine
GalN	D-galactosamine
G-CSF	Granulocyte colony-stimulating factor

GM-CSF	Granulocyte-macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid
HL-60	Human leukaemic cell line
Hsp	Heat shock protein
ICAM	Intracellular cell adhesion molecule
ICE	interleukin-1β converting enzyme
IFNγ	Interferon-γ
IκB	NFκB inhibitory proteins
IKK	IκB kinase
IL-1	Interleukin-1 etc.
ILA	inducible lymphocyte activator
iNOS	inducible nitric oxide synthase
$Ins(1,4,5)P_3$	Inositol 1,4,5-trisphosphate
IP_6	Inositol hexakisphosphate
Iscove's MDM	Iscove's Modified Dulbecco's medium
JNK	c-Jun N-terminal kinase
LPS	Bacterial lipopolysaccharide
LTα	Lymphotoxin-α
LTβ	Lymphotoxin-β
LTB_4	Leukotriene B_4
mAb	Monoclonal antibody
MACH	MORT1-associating CED homologue
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase

MAPKAP kinase	MAP kinase-activated protein kinase
MEK	MAPKK
MEKK	MAPKKK
$MgCl_2$	Magnesium chloride
mRNA	messenger RNA (q.v.)
mTNFα	Membrane-bound TNFα
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor-κB
NGF	Nerve growth factor
NIK	NFκB-inducing kinase
NK cell	Natural killer cell
NO	Nitric oxide
O_2^-	Superoxide anion
pAb	Polyclonal antibody
PAF	Platelet-activating factor
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-hydroxykinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PMA	Phorbol-12-myristate 13-o-acetate
PP	Protein phosphatase

PPP	Platelet poor plasma
PtdIns(3,4,5)P3	Phosphatidylinositol 3,4,5-trisphosphate
PTyrK	Protein tyrosine kinase
RIP	Receptor-interacting protein
SDS	Sodium dodecyl sulphate
SNAP	S-nitroso-N-acetylpenicillamine
SOD	Superoxide dismutase
sTNFα	Soluble TNFα
TGFβ	Transforming growth factor-β
TNFα	Tumour necrosis factor-α
TNF-BP	TNFα binding protein
TNFR	Tumour necrosis factor receptor
TNFR55	55 kD TNFR
TNFR75	75 kD TNFR
TRADD	TNFR55-associated death domain protein
TRAF	TNFR associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAMP	Tyrosine-rich acidic matrix protein
TRIP	TRAF-interacting protein
Tris	Tris[hydroxymethyl]-aminomethane

N.B. Standard abbreviations also used (eg mM for millimolar, etc)

Chapter 1: Introduction

1.1 The neutrophil

1.1.1 General structure, contents and origin

The neutrophilic polymorphonuclear leukocyte originates from the myeloid series in the bone marrow and is the most numerous of the circulating phagocytes, representing in most species 50 to 60% of the circulating white blood cell population. On electron microscopy this terminally-differentiated cell has a mean diameter of approximately 7 μm and is characterized by a multilobed chromatin-dense nucleus and distinctive cytoplasmic granules which distinguish it from other granulocytes. Neutrophil granules contain an array of enzymes, receptors and antimicrobial proteins which participate in the many functions of the cell. Traditionally, neutrophil granules were classified by their staining for peroxidase i.e. peroxidase-positive (azurophil or primary) or peroxidase-negative (specific or secondary); however this proved to be too simplistic to account for differential exocytosis of granule protein and incorporation of granule membrane into plasma membrane, hence a new classification was devised, sorting granules into four sub-populations (Borregaard et al., 1993).

1.1.1a Secretory vesicles

The secretory vesicle membrane contains the adhesion receptor CD11b/CD18 (Mac-1), which is essential for integrin-mediated neutrophil adhesion (Sengelov et al.,

1993a, Calafat et al., 1993), in addition to the *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) receptor (Jesaitis et al., 1982), alkaline phosphatase, and cytochrome b_{558} (the membrane component of the NADPH oxidase; Calafat et al., 1993); FcRIII receptors (for the Fc component of antibodies) and decay accelerating factor may also be present (Tosi and Zakem, 1992, Berger and Medof, 1987). These vesicles are the most readily and rapidly mobilised of the neutrophil granules (Sengelov et al., 1993b), allowing swift upregulation of plasmalemmal CD11b/CD18 (promoting adhesive events) and fMLP receptor numbers (augmenting cellular chemoattractant responsiveness).

1.1.1b Specific (secondary) granules

These spherical, oval or elongated granules are defined by their lactoferrin content; they are also an important source of cytochrome b_{558} (Borregaard and Tauber, 1984), adhesion molecules (Bainton et al., 1987) and fMLP receptors (Fletcher and Gallin, 1983).

1.1.1c Gelatinase (tertiary) granules

Smaller than specific granules, gelatinase granules constitute approximately 25% of all peroxidase-negative granules. They are mainly composed of the protease gelatinase, which may be involved in digesting the vascular basement membrane to allow neutrophil extravasation (Weiss and Peppin, 1986).

1.1.1d Azurophilic (peroxidase-positive) granules

Azurophilic granules store most of the proteolytic and bactericidal proteins (myeloperoxidase, cathepsins, elastase, β-glucuronidase, lysozyme, defensins etc.) and contain no receptors or adhesion molecules in their membranes. Mobilisation of these granules is slow and limited.

While it is likely that the majority of the constituents of neutrophil granules have evolved to promote rapid tissue migration and effective bacterial killing, it is apparent that inappropriate or excessive release of these agents would be highly toxic to host tissue. While the neutrophil does not contain large amounts of subcellular structures such as Golgi apparatus and mitochondria, it does however have significant endoplasmic reticulum content. The plasma membrane is of fundamental importance in this highly responsive cell since it houses the receptors which detect inflammatory events and signalling molecules and provides the link between the extracellular milieu and the intracellular environment for many receptor-mediated events including chemotaxis, phagocytosis, secretion, aggregation and the oxidative burst. An intimate communications network must therefore operate between the membrane and the secretory and specific granules which renew many major membrane components and also with the cellular cytoskeleton which lies mainly in the sub-membrane region and is responsible for the complex motility functions of the neutrophil.

Neutrophils are produced in the human bone marrow at a rate of 10^{11} cells per day (Cannistra and Griffin, 1988). Constitutive granulocytopoeisis is controlled by stromal cell-derived growth factors such as interleukin 3 (IL-3), and granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) which are lineage specific and direct the production and differentiation of bone marrow progenitor cells. These factors play an important role in the control of inflammation since their release is highly sensitive to cytokines such as IL-1 and TNFα which are generated at sites of inflammation; thus the rate of neutrophil differentiation can increase as much as 10-fold during the response to infection, inflammation or stress (Cannistra and Griffin, 1988). Mature neutrophils retained within the bone marrow constitute an important reservoir of cells which can be stimulated to egress into the bloodstream (e.g. by a decreased neutrophil count in blood perfusing the marrow) where they distribute between circulating and 'marginating pools' of neutrophils temporarily sequestered in the microvascular beds of the lung, spleen and liver. Circulating and marginating pools can exchange with each other, however little is understood about the fate or size of these interrelated tissue pools (MacNee and Selby, 1990).

Studies using radiolabelled neutrophils have indicated that they have a short half-life (approximately 4 hr) in the blood (Price and Dale, 1977) and although it is unclear why these cells turn over so rapidly in healthy subjects, this may be related to their role in immuno-surveillance and/or non-immunological roles in maintaining homeostasis.

1.1.2 Neutrophil functions

Neutrophils form part of the natural (non specific) immune system and are the first cells to be summoned to sites of infection or injury (Schleimer et al., 1989) where their primary role is to phagocytose and destroy invading micro-organisms. Accordingly, neutrophils have evolved a variety of rapid, and co-ordinated responses which allow them to reach an area of inflammation and react appropriately to the inciting agent. For example, neutrophil motility requires regulation of adhesion and cytoskeletal reorganization. Upon recognition of a foreign agent, phagocytosis, secretion of pre-formed proteolytic enzymes and bactericidal proteins, and de novo production of reactive oxygen intermediates ensues. Collectively these are termed the 'effector' responses of neutrophils, however they also limit microbial growth thereby allowing time for adaptive (specific) immunological responses to develop (Mannion et al., 1990). With an excessive number of microbes, however, neutrophil defenses are ineffective in the absence of opsonins and various agents that amplify the inflammatory response which emphasises the co-operative interaction between natural and adaptive components of the immune system.

Cationic proteins and proteolytic enzymes, stored within the cytoplasmic granules, are released into phagolysomes and/or extracellulary (Borregaard et al., 1993). Reactive oxygen intermediates are produced by the NADPH oxidase, a multicomponent enzyme complex which transfers electrons from NADPH to

molecular oxygen to generate superoxide anions. Neutrophil targets include bacteria (particularly streptococci), fungi, protozoa, viruses, virally infected cells and tumour cells (Ratcliffe et al., 1988).

The fundamental importance of the neutrophil in defense is illustrated by the marked propensity of the host to develop infection in conditions of deficiency in number and/or function. Examples of these disorders include neutropaenia induced by chemotherapeutic agents, chronic granulomatous disease, where components of the NADPH oxidase are absent or defective (Smith and Curnutte, 1991), leukocyte motility disorders, where the regulation of cytoskeletal events required for motility is abnormal (Howard et al., 1994), leukocyte adhesion disorders, where adhesion molecule functions are deficient (Albelda et al., 1994) and disorders of granule secretion such as the Chediak-Higashi syndrome (Wolff et al., 1972).

1.1.3 Role of the neutrophil in inflammation

The initial phase in an acute inflammatory response is recruitment of neutrophils to the infected site by chemotactic agents which induce polarization and directed locomotion along a chemotactic gradient. A foreign agent, such as a bacterium, may drive chemotaxis by a variety of mechanisms: (i) directly, by the release of chemotactic peptides from its surface (e.g. fMLP), (ii) via complement activation and C5a generation, or (iii) by induction of macrophage production of leukotriene B_4 (LTB_4), C-X-C chemokine family members such as IL-8 (Baggioloini et al., 1989),

or other cytokines such as TNFα and IL-1 which further amplify neutrophil recruitment by the secondary stimulation of other local cells to produce chemokines. Since neutrophils themselves are an important source of chemokines, including IL-8, the opportunity exists for autoregulation of further neutrophil migration (Kunkel et al., 1995).

Neutrophil arrest in the microcirculation initiates the interaction between the leukocyte and the endothelial cells and is a pre-requisite for endothelial transmigration. In most tissues, neutrophil sequestration and emigration occurs primarily within the post-capillary venules (Shaw, 1980, Downey et al., 1993, Doerschuk et al., 1994), however in the lung, the principal route of leukocyte retention, margination and emigration is in the pulmonary capillary (Schmid-Schonbein et al., 1980). In the systemic microvasculature, sequestration results as a consequence of the increased expression/activation of neutrophil surface adhesion molecules, however in the pulmonary circulation, alterations in neutrophil rheological properties, especially a reduction in their deformabilty, may also play an important contributory role. A variety of chemotactic factors have been shown to induce a modest reduction in neutrophil deformability which directly correlates with vascular sequestration (Worthern et al., 1989, Drost et al., 1992).

Neutrophils first roll or loosely tether themselves to the microvasculature wall via low-affinity interactions mediated by the selectin family of adhesion molecules, in particular L-selectin (CD62-L) on the neutrophil and E- and P-selectins (CD62-E and

CD62-P respectively) on the endothelial cell (Bavilacqua and Nelson, 1993, Albeda et al., 1994). This process does not necessarily result in neutrophil transmigration however, and chemoattractant-mediated activation of β_2-integrin adhesion molecules, in particular CD11b/CD18 (Mac-1) (Kishimoto et al., 1989, Jutila et al., 1989), and the subsequent high-affinity binding of neutrophils to endothelial intracellular adhesion molecules (ICAM-1 and ICAM-2) must occur for subsequent neutrophil translocation to a site of inflammation (Smith et al., 1989, Dustin et al., 1986).

Under the influence of a chemotactic gradient, generated both locally and by diffusion of chemoattractants from the inflammatory focus, neutrophils emigrate between the endothelial cell layer of the microvascalture wall, penetrate the basement membrane and migrate through the interstitium toward the area of infection (diapedesis) where they finally adhere to extracellular matrix components such as laminin and fibronectin (Nathan and Sanchez, 1990, Cronstein and Weissmann, 1993). A wide variety of adhesion molecules have been identified and characterized on phagocytic cells (Cronstein and Weissmann, 1993).

On arrival at the inflammatory focus, the primary function of the neutrophil is the recognition, phagocytosis and destruction of pathogens. Phagocytosis involves two stages; recognition and internalization of the inciting agent into the phagosome and subsequent killing/neutralization and digestion of the foreign material. The neutrophil must first recognize a foreign particle before engulfment can occur and in some instances this is mediated by direct binding to materials such as

lipopolysaccharide (LPS) on the surface of the foreign organism. In most cases however, the particle must be opsonized by plasma binding proteins which may be specific (immunoglobulin) or non-specific (e.g. complement, fibronectin, C-reactive protein) to allow neutrophil receptor-mediated recognition (i.e. Fc and complement receptors, CR1 and CR3). For engulfment of an opsonized particle to occur, pseudopods rich in filamentous actin must form and surround the particle; this process requires actin polymerization and is therefore blocked by agents such cytochalasins (Zigmond and Hirsch, 1972). During phagocytosis, there is intra-phagosomal release of toxic oxygen metabolites (1.1.3a) which requires the initiation of an intense burst of oxygen consumption (Baldridge and Gerard, 1933), known as the respiratory burst, and cytosolic granules fuse with the invaginating plasma membrane (1.1.1) creating a highly toxic microenvironment. Other metabolic pathways are also activated by phagocytosis (e.g. Della Bianca et al., 1993) including the synthesis of bioactive lipids such as platelet-activating factor (PAF) and LTB_4 (Clancy et al., 1983, Chilton et al., 1984) and a number of cytokines (1.1.3b).

1.1.3a Production of reactive oxygen intermediates

The net reaction of the respiratory burst is the transfer of electrons from NADPH to oxygen, producing superoxide anions:

$NADPH + H^+ + 2O_2 \rightarrow NADPH^+ + 2H^+ + 2O_2^-$

NADPH produced by the cytosolic hexose monophosphate shunt functions as an electron donor to effect a one electron reduction of each of two atoms of molecular oxygen (Babior et al., 1973). The O_2^- formed dismutates to form hydrogen peroxide, a reaction catalysed by superoxide dismutase:

$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$

In the presence of halide ions (preferentially chloride ions) and H_2O_2, myeloperoxidase released from neutrophil azurophil granules can catalyse the generation of hypohalous acids, such as hypochlorous acid:

$H_2O_2 + HCl \rightarrow HOCl + H_2O$

The cytotoxic effects of hypochlorous acid include: oxidation/reduction of membrane proteins, leading to increased bacterial cell permeability (Albrich et al., 1986), oxidation of components of the bacterial respiratory chain (Rakita et al., 1989), membrane peroxidation (Winterbourn et al., 1992) and the formation of chloramine (Bernofsky, 1991). Additional reactive products such as hydroxyl radical and singlet oxygen may also be formed, but as a consequence of their short longevity, detection is difficult and their role is uncertain (reviewed in Rosen et al., 1995).

1.1.3b Cytokine production

Protein synthesis by neutrophils can be elicited by a variety of agonists including fMLP, TNFα and GM-CSF (Beaulieu et al., 1992), and several cytokines are produced including IL-1 (Tiku et al., 1986), IL-6 (Cicco et al., 1990), IL-8 (Bazzoni et al., 1991) G-CSF and M-CSF (Lindemann et al., 1989), and TNFα (Dubravek et al., 1990). Since a large number of neutrophils are recruited to inflammatory foci, the impact of cytokine synthesis by these cells may be highly significant.

1.1.4 Neutrophil-mediated tissue injury

While neutrophils are essential for host defense they have also been implicated in the pathophysiology of a variety of inflammatory conditions such as the adult respiratory distress syndrome (ARDS) (reviewed by Donnelly and Haslett, 1992), pulmonary fibrosis (e.g. Behr et al., 1991), vasculitic diseases (reviewed by Savage and Rees, 1994), rheumatoid arthritis (Robinson et al., 1992) and ischemia-reperfusion injury (reviewed by Williams, 1994). Neutrophil-derived hydrolytic enzymes and oxidatively inactivated protease inhibitors have been detected in fluids isolated from inflammatory sites (Weiss, 1989). In addition, it has been postulated that chronic activation of neutrophils may also initiate tumour development since some reactive oxygen intermediates cause DNA damage *in vitro* (Weitzman and Gordon, 1990) and proteases promote tumour cell migration (Opdenakker and Van Damme, 1992).

Under normal conditions neutrophils can migrate to sites of infection without damaging host tissue. The secretory processes activated during this process (e.g. to enable the expression of adhesion molecules and to activate signalling responses to chemoattractants; Borregard et al., 1993) however are also linked to the activation of microbicidal activity.

Neutrophil-mediated host tissue damage may arise through several independent mechanisms. These include (i) premature activation during migration, (ii) extracellular release of microbicidal agents during 'frustrated phagocytosis' where micro-organisms are either too large for full phagocytosis to occur, or evade this process, (iii) removal of infected or damaged host cells and debris as an initial step in tissue remodeling, (iv) overwhelming of the normal controls preventing generalised activation, or (v) failure to terminate the acute inflammatory response.

1.1.5 Neutrophil priming

Priming refers to the process whereby an agent which has no secretory effect on its own causes a dramatic upregulation of the response of the neutrophil to a secondary stimulus (figure 1.1.5). Neutrophil priming was first reported by Guthrie and co-workers (1984) who demonstrated that pre-incubation of freshly isolated human neutrophils with LPS did not in itself elicit a respiratory burst, but greatly potentiated the oxidative response to fMLP, and to a far lesser extent than that observed with PMA. Minimal superoxide anion generation was recorded in unprimed cells

stimulated with fMLP; thus in view of the fact that neutrophil preparative methods themselves may induce a degree of cell priming (Haslett et al., 1985), truly unprimed neutrophils in the circulation are likely to be entirely unresponsive to fMLP (with respect to NADPH oxidase activation) if not exposed to a priming agent. A wide array of physiological substances have been shown to act as priming agents and these may be derived from macrophages (e.g. GM-CSF, TNFα), endothelial cells (PAF, TNFα) or bacteria (e.g. LPS). The pre-incubation times required to obtain a maximally primed response range from minutes (e.g. PAF, TNFα; Vercellotti et 1988, Berkow et al., 1987) to a few hours (LPS, GM-CSF; Guthrie et al., 1984, Weisbart et al., 1986), implying that these agents mediate neutrophil priming via distinct signal transduction routes. Moreover, a thorough understanding of the mechanism of priming is further complicated by the fact that sub-maximal concentrations of classical 'activating' agents, such as fMLP, can also serve to prime neutrophils (Bender et al., 1983), whereas the archetypal priming agent TNFα can cause direct triggering of degranulation and respiratory burst activity in adherent neutrophils (Nathan, 1987, Richter et al., 1989, Nathan et al., 1989, Dri et al., 1991).

In addition to the respiratory burst, priming of the degranulation response (Fittschen et al., 1988) and of the generation of lipid mediators (principally arachidonic acid, LTB_4 and PAF; Doerfler et al., 1989, 1994, DiPersio et al., 1988) have been described, and priming has been demonstrated to be tightly linked with both shape change (Haslett et al., 1985) and adhesion molecule (CD11b/CD18) expression and activity (Condliffe et al., 1996).

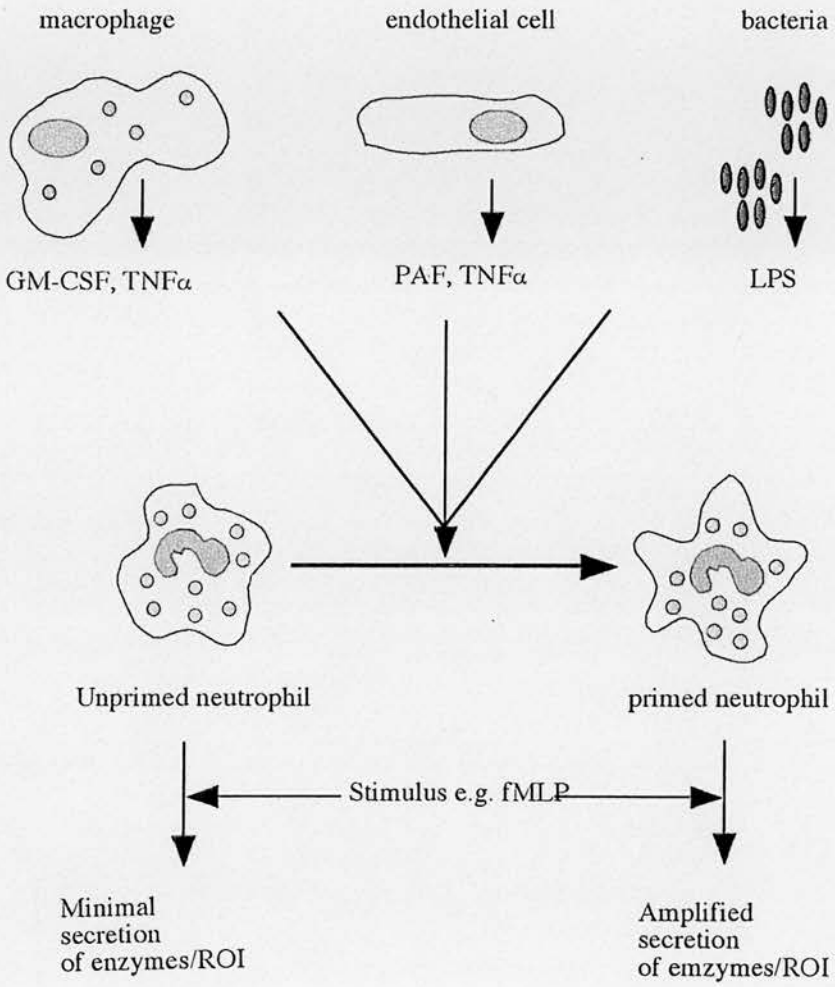


Figure 1.1.5 Neutrophil priming

Priming of neutrophils by the products of activated macrophages (e.g. TNFα, GM-CSF), endothelial cells (e.g. PAF) and bacteria (e.g. LPS) does not lead directly to the release of toxic products but results in amplified secretion of reactive oxygen intermediates and enzymes when a further stimulus (e.g. fMLP) is encountered.

The capacity for neutrophils to recover from a primed state was recently demonstrated by our group (Kitchen et al., 1996b) where, in contrast to the very protracted effects of TNFα, LPS and GM-CSF, incubation of neutrophils with a maximal concentration of PAF induced rapid but transient receptor-mediated priming with full recovery of cells to a state where only basal unprimed levels of fMLP-stimulated superoxide anion generation, shape change and CD11b/CD18 activity was observable. Moreover, these cells had the ability to fully 're-prime' upon subsequent stimulation with PAF or TNFα.

1.2 Apoptosis

It has now well established that the death of nucleated cells can be classified into two distinct types, necrosis, or 'accidental' cell death, and apoptosis, or 'programmed' cell death (Kerr et al., 1972, Wyllie et al., 1980, Duvall and Wyllie, 1986). Apoptosis occurs where death is predictable or planned, or where cell turnover is physiologically rapid e.g. the elimination of autoreactive T-cells (Jenkinson et al., 1989), involution of cells deprived of necessary growth factors (Duke and Cohen, 1986), morphogenetic death of cells during embryonic and early post-embryonic development (Glucksmann, 1951), and in the killing of cells which serve as targets for T, NK or antibody-dependent cell-mediated cytotoxic mechanisms (Duke et al., 1983).

During apoptosis, ultrastructural studies have shown that, in contrast to necrosis, the cells shrink due to cytoplasmic condensation, and in some cell types, detachment of cytoplasmic 'blebs' occurs; despite this, the plasma membrane stays intact and the cells retain the ability to exclude vital dyes. Dramatic changes in the cell surface also occur, such as exposure of phosphatidyl serine, loss of microvilli, and the development of invaginations (Morris et al., 1984, Fadok et al., 1992). Organelles such as mitochondria and cytoplasmic granules remain intact although the endoplasmic reticulum may dilate. Nuclear changes, however, are the most characteristic features of apoptosis: the chromatin rapidly condenses into dense crescent-shaped aggregates at the periphery of the nucleus, which if multilobed, may condense into a single spherical 'pyknotic' body.

The 'biological hallmark' of apoptosis is internucleosomal cleavage of chromatin (Wyllie, 1980) whereby an endogenous endonuclease fragments DNA at linker sites between nucleosomes to generate low molecular weight fragments of chromatin which are integer multiples of 180-200 base pairs of DNA associated with a nucleosome (Arends et al., 1990).

Apoptotic cells are thereafter ingested and degraded very rapidly by phagocytes, particularly macrophages, *in vivo*; so that in tissue sections apoptotic cells are usually seen to be within other phagocytes (Alison and Sarraf, 1992. Furthermore, in these examples, apoptosis is not associated with any evidence of local tissue injury or the induction of an inflammatory response, suggesting that apoptosis may represent a

tissue-injury limiting mechanism for the removal of senescent cells which has very direct and obvious implications for the removal of granulocytes from an inflammatory focus.

1.2 1 Neutrophil apoptosis

It is generally accepted that neutrophils meet their fate at the inflamed site with little if any evidence to suggest that extravasated neutrophils return to the blood stream either directly or via the lymphatic drainage system (Haslett and Henson, 1988). In the face of such observations it was assumed previously that neutrophils present at an inflammatory focus died by necrosis and that the cell debris was removed by local macrophages (Hurley, 1983). However, neutrophils contain a variety of potentially injurious agents (Henson and Johnson, 1987, Weiss, 1989) including proteases and other enzymes with the capacity both to damage tissue directly (Weiss, 1989) and to cleave matrix proteins into chemotactic fragments (Vartio et al., 1981), and hence disintegration of these cells would serve to amplify the inflammatory response and recruit even more inflammatory cells. Moreover, phagocytic clearance of such neutrophil debris would be expected to cause an additional pro-inflammatory response by the macrophage.

The classical lectures of Metchnichoff (1891) showed that in vital preparations of inflamed tissue there was evidence of an alternative fate for the neutrophil whereby seemingly intact cells were removed by macrophages; however, the significance of

these observations were overlooked for many years. Almost a century later, *in vitro* experiments using cells purified from human peripheral blood demonstrated recognition and phagocytosis of neutrophils when aged in culture for 24 hr by mature monocyte-derived macrophages (Newman et al., 1982). This process generated interest as a possible tissue injury-limiting mechanism and further studies revealed that the change in neutrophil phenotype which promoted recognition and phagocytosis of these intact senescent cells was neutrophil apoptosis (Haslett et al., 1987, Savill et al., 1989a).

Subsequent experimental data obtained again using peripheral blood neutrophils demonstrated that these cells undergo constitutive apoptosis *in vitro* with the majority of neutrophils dying by this process after 24 hr in culture (Begley et al., 1986, Lopez et al., 1986, Colotta et al., 1992, Lee et al., 1993). This process was associated with maintenance of membrane integrity, down-regulation of neutrophil functions such as chemotaxis and phagocytosis, hypo-responsiveness to secretagogue signals (Whyte et al., 1993a), and the capacity to be phagocytosed intact by macrophages (Savill et al., 1989a and b, 1990b, 1992, Haslett et al., 1989, Whyte et al., 1993a).

The usual response of the macrophage to phagocytosis of particles *in vitro* is the release of inflammatory mediators (e.g. thromboxanes, enzymes and cytokines), however the ingestion of apoptotic neutrophils does not excite a pro-inflammatory response (Meagher et al., 1992). Human monocyte-derived macrophage recognition

of apoptotic neutrophils has been demonstrated to involve the macrophage αvβ3 (vitronectin receptor) and CD36 (thrombospondin receptor) which appear to co-operate with thrombospondin to bridge the macrophage to the apoptotic cell (Savill et al., 1990b, 1992a) by a process which appears to be independent of new protein synthesis (Whyte et al., 1997). Upregulation of thrombospondin-mediated phagocytosis of cells undergoing apoptosis *in vitro* has been observed with pro-inflammatory cytokines (GM-CSF, IL-1β, interferon-γ (IFNγ), TNFα) and by transforming growth factor (TGF)-1β which has both pro- and anti-inflammatory properties (Ren and Savill, 1995). Finally, 'semi-professional' phagocytes, such as mesangial cells and fibroblasts, have the capacity to recognize and ingest apoptotic neutrophils *in vitro* (Savill et al., 1990a, Hall et al., 1994) which may provide support for the macrophage system *in vivo*.

1.2.3 Modulation of neutrophil apoptosis

While the mechanisms regulating neutrophil survival and death are poorly understood, there is now considerable evidence to suggest that this process is not immutable since the rate at which these cells undergo apoptosis, at least *in vitro*, can be altered. For example, a wide variety of pro-inflammatory mediators such as LPS, C5a, GM-CSF, IL-1β, IFNγ and LTB_4 have been shown to increase the survival of these cells (Begley et al., 1986, Brach et al., 1992, Cox et al., 1992, Colotta et al., 1992, Lee et al., 1993). Furthermore, the demonstration that LPS-mediated inhibition of neutrophil apoptosis results in prolongation of neutrophil functional

longevity, as assessed by secretion and chemotaxis, suggests that certain agents may upregulate neutrophil function both by activating and/or priming for enhanced functional responsiveness to secretagogue agonists and by delaying apoptosis (Lee et al., 1993), as illustrated in figure 1.2.3A. While the mechanisms regulating cytokine-mediated modulation of neutrophil apoptosis are poorly understood, there is data to support a role for tyrosine phosphorylation and intracellular acidification in GM-CSF and G-CSF-mediated inhibition of cell death respectively (Yousefi et al., 1994, Gottlieb et al., 1995).

Although it is well established that the longevity of neutrophils can be altered by the inhibition of apoptosis, these cells appear highly resistant to any attempt to accelerate this process using physiological agonists. Indeed, even experimental stimuli that are potent stimulators of apoptosis in other cell types, such as elevation of intracellular Ca^{2+}, extreme hypoxia and high dose corticosteroids, all inhibit rather than augment apoptosis in neutrophils (Whyte et al., 1993b, Hannah et al., 1995, Cox, 1995, Meagher et al., 1996).

While it is clear that neutrophil apoptosis can be induced pharmacologically e.g. by inhibition of protein kinase C (PKC; Cousin et al., 1997, MKB Whyte, Ph. D Thesis, 1993), or by treatment with the protein synthesis inhibitors cycloheximide or actinomycin D (Brach et al., 1992, Whyte et al., 1997), controversy exists in the literature regarding the ability of physiological agonists to stimulate apoptosis in these cells. While one study reported that IL-6 induced neutrophil apoptosis (Afford

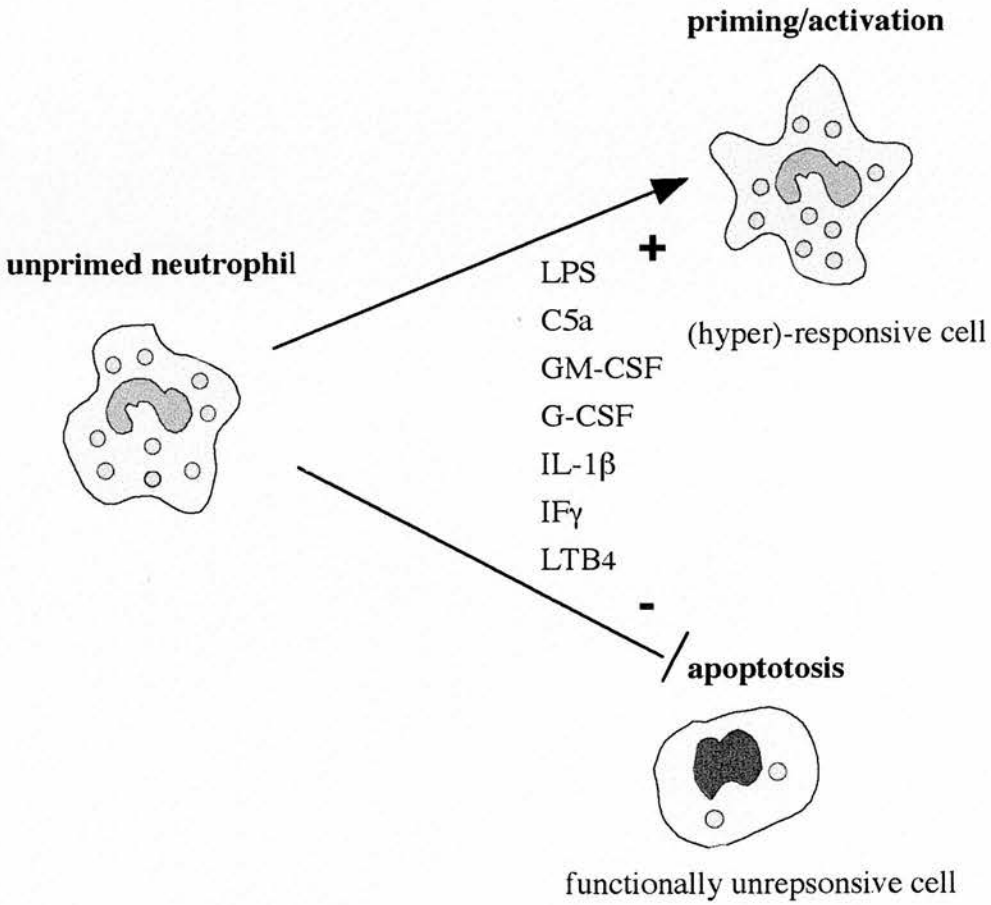


Figure 1.2.3 Modulation of neutrophil apoptosis
Certain agents may upregulate neutrophil function by activation and/or priming for enhanced functional responsiveness to secretagogue agonists and by delaying apoptosis.

et al., 1992), this effect was small, has not been reported by at least two other groups (Takeda et al., 1993, Biffl et al., 1995), and the extent of constitutive apoptosis recorded (10% cf. 15% in the presence of IL-6 at 24 hr) was very different to that reported in all other comparable studies.

One cytokine of particular interest is TNFα which is a powerful priming agent in neutrophils yet has the capacity to induce apoptosis in a variety of cell types including T-lymphocytes (Zheng et al., 1995) and HL-60 cells (Obeid et al., 1993). The data from studies regarding the effects of TNFα on neutrophil longevity are conflicting however, as they suggest that TNFα either inhibits (Colotta et al., 1992), has no effect on (Kwon et al., 1988), or induces apoptosis (Takeda et al., 1993) in these cells.

Apoptosis thus not only determines the functional longevity of granulocytes but can itself be modulated and controlled by external mediators with relevance to the control of inflammation. Hence, in contrast with necrosis, it provides a neutrophil removal mechanism which can also be influenced by inflammatory mediators. These data all predict that this process plays an important role in the 'safe' disposal of intact but effete neutrophils from an inflamed focus as illustrated in figure 1.2.3B. This view is supported by recent demonstrations of this process occurring *in vivo*, for example in endotoxin-induced experimental lung injury (Cox et al., 1995), the neonatal respiratory distress syndrome (Grigg et al., 1991) and in experimental glomerulonephritis (Savill et al., 1992b).

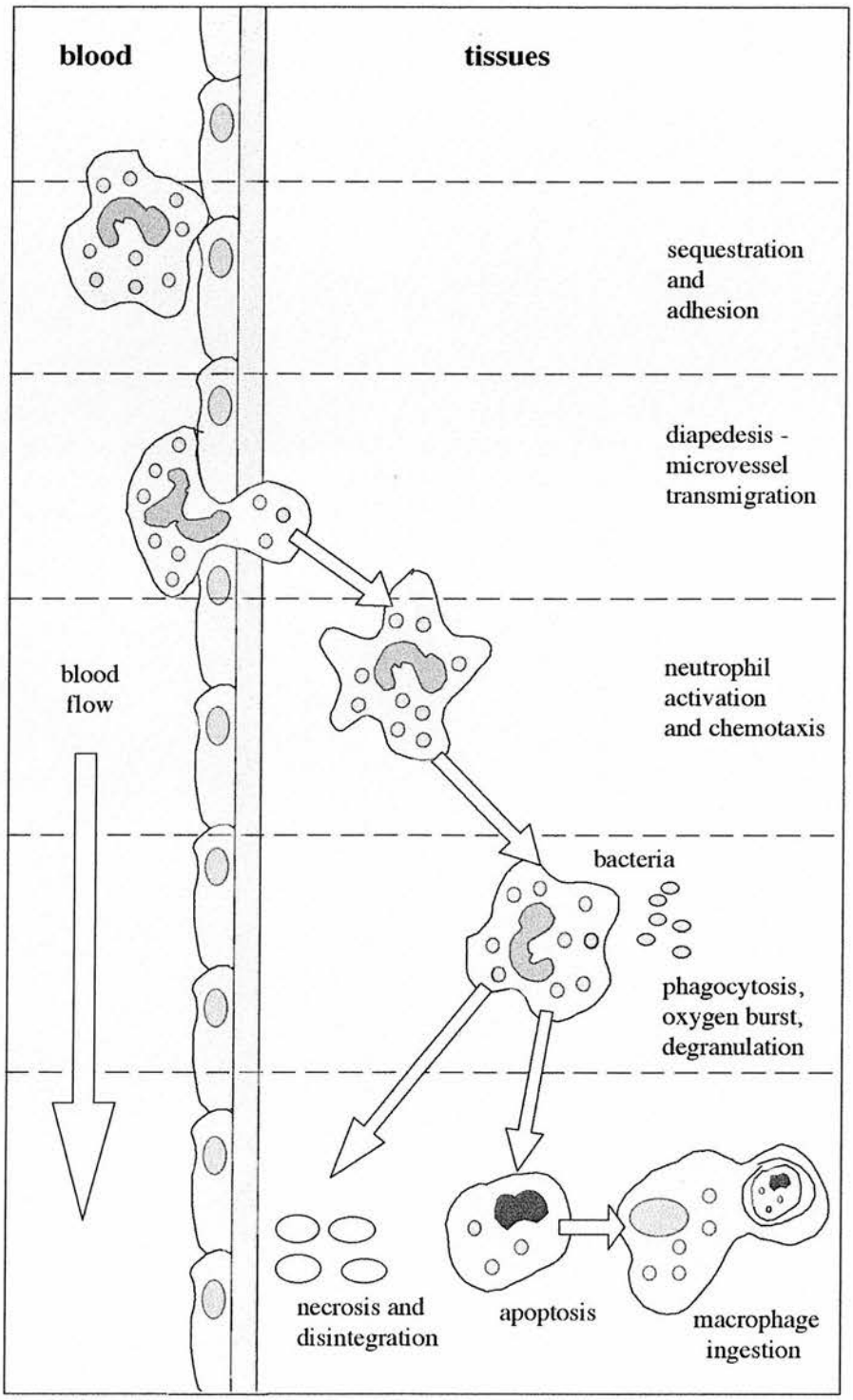


Figure 1.2.3B The role of the neutrophil in inflammation

1.3 TNFα

1.3.1 Historical background

The first person to exploit the phenomenon of induction of haemorrhagic necrosis of tumours during some coincidental pyrogenic bacterial infections was the New York surgeon William Coley (1862-1936). Coley used preparations of Gram-positive and Gram-negative bacteria for cancer therapy and claimed successes in some cases (Coley, 1906), however the side effects of his treatment were intolerable and these early attempts at therapy were stopped. Much later LPS was isolated as the active component in comparable bacterial preparations to those administered by Coley (Shear and Andervont, 1936, Hartwell et al., 1943, Kahler et al., 1943, Shear et al., 1943a and b, Shear, 1944) and the presence of an agent which was cytotoxic to tumours in serum of Bacille Calmette-Guerin (BCG)-infected and LPS-challenged animals was demonstrated by transfer experiments (O'Malley et al., 1962, Carswell et al., 1975). This substance, which induced necrosis of the meth-A sarcoma in mice, also killed transformed cells *in vitro*, including WEHI 164 cells (Espevik and Nissen-Meyer, 1986). This otherwise undefined and unpurified substance was named tumour necrosis factor.

In a parallel line of research, Cerami and co-workers, who were investigating the underlying cause of wasting in chronic infectious disease, observed that rabbits infected with Trypanosoma brucei developed severe cachexia, losing up to 50% of their live weight, despite a low parasitic load. Paradoxically, the final stage of

wasting was marked by a profound accumulation of triglycerides in the serum which was attributed to a systemic deficiency in the activity of lipoprotein lipase (Rouzer and Cerami, 1980). The factor responsible for metabolic suppression of this enzyme was also induced by LPS in certain endotoxin-sensitive mouse strains (C3H/HeN) and moreover, this activity was transferable to LPS-resistant strains (C3H/HeJ; Kawakami and Cerami, 1980). The ability of this factor to completely block lipase activity was exploited in the development of a sensitive bioassay (Kawakami et al., 1982) for a molecule that was subsequently referred to as 'cachectin' (see Beutler, 1992, Beutler and van Huffel, 1994). Endotoxin-stimulated macrophages were found to be a major source of the TNF and cachectin activities (Mannel et al., 1980, Torti et al., 1985) and cachectin was found to be potently cytotoxic for WEHI 164 cells *in vitro*, indicating a high degree of homology between these molecules (Beutler et al., 1985). Molecular cloning and expression finally established that cachectin and TNF were in fact the same molecule (Pennica et al., 1984, Wang et al., 1985).

TNF became known as TNFα when a third route of research led to the discovery of lymphotoxin (LT), which was referred to initially as TNFβ. LT was identified in studies investigating the cytotoxic factors responsible for so-called 'by-stander' killing (Granger and Williams, 1968) and the potential mediators of tissue damage in delayed-type hypersensitivity (Ruddle and Waksman, 1967, 1968a, b) which focused on a factor produced by T lymphocytes following antigenic or mitogenic stimulation. The relationship of LT with TNF/cachectin was only determined after complete sequencing of the molecule (Pennica et al., 1984, Gray et al., 1984). It now appears

that much of the biological activity originally attributable to T cell LT was probably derived mostly from TNFα, however it only became apparent subsequently that T cells, as well as macrophages, produced TNFα (Kinkhabwala et al., 1990).

LT and TNFα share about 30% homology in their primary amino acid sequence; of greater significance is the observation that these regions of sequence homology allow for similarity in the tertiary and quaternary structure of the two molecules (Pennica et al., 1984). LT, like TNFα, forms a trimeric structure (Eck et al., 1992) and is sufficiently structurally conserved to bind to the same receptors as TNFα (TNFR55 and TNFR75; section 1.3.6) with similar affinity (Loetscher et al., 1991), although the cellular response to this binding appears different (Schuchmann et al., 1995). While LT lacks a hydrophobic transmembrane domain and is entirely secreted (section 1.3.8), membrane LT has been detected on the surface of activated B and T cells as well as T cell hybridomas, but in association with a 33 kDa glycoprotein (Ware et al., 1992, Browning et al., 1991) identified subsequently as a member of the TNF/NGF receptor family ligands (section 1.3.7) and named LTβ; thus the original LT is now known as LTα. The three related proteins are now universally referred to as TNFα, LTα and LTβ.

1.3.2 Source and structure of TNFα

The human TNFα gene is located on the short arm of chromosome 6 within the Class III locus of the major histocompatibility complex (MHC) genes (Nedwin et al.,

1985). About 70% of the amino acid sequence of TNFα was found to be conserved when TNFα from nine mammalian species were compared. TNFα is expressed as a 26 kDa pre-peptide which is proteolytically cleaved to release the 17 kDa mature protein of 157 amino acid residues from the cell surface (Kreigler et al., 1988). The membrane-bound disintegrin metalloproteinase that processes precursor TNFα has now been cloned and named TACE (for 'TNFα-converting enzyme'; Moss et al., 1997). Both the secreted 17 kDa protein and the 26 kDa pre-peptide spontaneously trimerize and form the bioactive ligand (Smith and Baglioni,, 1987). The three-dimensional structure of the trimer has been determined (Eck et al., 1988) and a model of ligand-receptor interaction deduced (Eck et al., 1992).

Mainly for historical and technical reasons, active TNFα was (and still is by some) considered to act mostly as a (secreted) soluble protein, however this view is now changing. Treatment of mice with a metalloprotease inhibitor protected the animals injected with a lethal dose of LPS from endotoxic shock (Mohler et al., 1994) which suggests that it is secreted, not membrane-bound TNFα (mTNFα) that is the key player in this pathological process. By 1988, mutant cell lines unable to secrete TNFα were described and in this context, mTNFα proved to be as active as the soluble forms in terms of cytotoxicity and anti-tumour activity (Kriegler et al., 1988, Perez et al., 1990). Moreover, mTNFα has recently been demonstrated to have a pro-apoptotic effect on WEHI 164 cells (Monastra et al., 1996). Another report has demonstrated that engagement of TNFR55 and TNFR75 by mTNFα under cell-cell contact conditions is able to trigger much more dynamic and perhaps qualitatively

different effects than secreted TNFα and the authors suggest that the mTNFα may be the prime activating ligand of TNFR75 (Grell et al., 1995). Taken together, these findings suggest that secreted TNFα may only be observed under exceptional physiological or pathophysiological conditions, perhaps triggered under circumstances such as in massive bacteraemia, while mTNFα may play a more important physiological role at much lower concentrations and be under careful regulation at the levels of transcription, translation and secretion.

TNFα is produced principally by monocytes and macrophages in response to LPS, however other sources of this cytokine include lymphocytes, fibroblasts, mast cells and even neutrophils themselves (Dubravev et al., 1987). TNFα biosynthesis is regulated at two levels, transcription and translation, which work in concert with each other. The activation of macrophages by LPS causes a 50-fold increase in TNFα messenger RNA and a 100-fold increase in translational efficiency. As a result, the corresponding rate of TNFα protein production can increase by a factor of several thousand. TNFα acts in concert with various other cytokines, in particular, its activity parallels closely that of the interleukins IL-1 and IL-6.

1.3.3 The biological role of TNFα

TNFα plays a critically important role in mediating a number of physiological immune events, including temperature regulation, bone and cartilage remodeling, haematopoiesis and the regulation of extracellular matrix production. In addition,

TNFα represents an integral component of normal host defense as it augments immune cell antimicrobial, antiviral and tumouricidal activity, enhances neutrophil adherence to endothelium, and is crucial to granuloma formation in response to intracellular pathogens. Moreover, TNFα serves as a major host-derived inducer of other bioactive mediators, including chemotactic and activating polypeptides and lipids, and can further amplify its own biological effects by acting in an autocrine or paracrine manner to stimulate secretion of additional TNFα from immune or non-immune cells. Hence, TNFα displays a broad spectrum of biological activities.

Many aspects of TNFα as a mediator of inflammation have been attributed to its ability to influence neutrophil function. TNFα serves to upregulate many neutrophil activities including priming for enhanced responsiveness to secretagogue agonists (Klebanoff et al., 1986, Shalaby et al., 1985, Atkinson et al., 1988), promotion of adherence to endothelial cells and surfaces coated with extracellular matrix proteins (Gamble et al., 1985, Dri et al., 1991, Thompson and Matsushima, 1992), inhibition of chemotactic responsiveness to fMLP (Atkinson et al., 1988), stimulation of phagocytosis in response to both opsonized and unopsonized zymosan (Shalaby et al., 1985, Klebanoff et al., 1986) and enhancement of zymosan-induced LTB_4 release (Petersen et al., 1990). In addition, TNFα causes direct triggering of degranulation and respiratory burst activity in adherent cells (Nathan, 1987, Richter et al., 1989, Nathan et al., 1989, Dri et al., 1991).

1.3.4 The role of TNFα in disease

As a result of the high toxicity of TNFα in animals and humans this cytokine did not fulfill initial expectations that it may prove to be of benefit in the treatment of cancer. The tumouricidal action of TNFα is complex and may be direct or mediated through its effects on endothelium and immune effector cells, depending on the system (Fiers, 1993). The haemorrhagic necrosis of tumours by TNFα results in part from the activation of endothelium and consequent pro-coagulation effects (Bevilacqua et al., 1986). The activation of neutrophils, monocytes, and lymphocytes (NK cells) can also contribute to the anti-tumour effect of TNFα. However, stimulation of these immunoregulatory cells and endothelium also leads to marked pro-inflammatory effects which have been manifested in human clinical trials by fever, dose-limiting hypotension, hepatotoxicity, pulmonary oedema, thrombocytopenia, intravascular thrombosis and haemorrhage (Hauser et al., 1990, Kilbourn et al., 1990, Van Der Poll et al., 1992, Van Ostade et al., 1993). It is these pro-inflammatory side effects which have severely limited the use of systemically administered TNFα in patients with malignancy (Jones and Selby, 1989, Taguchi and Sohmura, 1991).

Although TNFα is vital to the maintenance of immune homeostasis, the beneficial effects of this cytokine usually predominate when small immunologically active amounts are produced in tissues during host defense (Tracey et al., 1989). In contrast, the over zealous production of TNFα can trigger circulatory shock and severe tissue injury (Tracey et al., 1989, Tracey, 1992). Numerous studies have demonstrated that TNFα mediates many of the pathological events induced by

endotoxin which culminate in cardiovascular collapse; these include hypotension, intravascular thrombosis, and severe pulmonary oedema and haemorrhage (Remick et al., 1990) all of which can be prevented by prophylactic administration of anti-TNFα antibodies (Tracey et al., 1987). Dysregulated TNFα activity has now been reported in the pathogenesis of many disease states, some of which are listed in table 1.3.4.

Disease	**Complications**
Septic shock	Hypotension and tissue injury
ARDS	Increased lung permeability
Cerebral malaria	Cerebral inflammation and infarction
Systemic lupus erythematosus	Tissue injury and renal failure
Rheumatoid arthritis	Inflammation and acute phase responses
Diabetes mellitus	Beta cell cytotoxicity
AIDS	Activation of latent infection
Cancer	Anaemia and whole-body protein loss (cachexia)

Table 1.3.4 Diseases in which dysregulated TNFα activity has been implicated (Adapted from Tracey, 1995)

It is now well established that inter-individual variation exists with respect to TNFα production by peripheral blood mononuclear cells isolated from normal volunteers (Molvig et al., 1988), and such differences have been genetically linked to HLA type and to other polymorphic markers. Of interest, a recent study demonstrated that

possession of a genetic variant in the promoter of the TNFα gene (termed the TNF2 allele), which is associated with higher constitutive and inducible concentrations of TNFα transcription, conferred increased susceptibility for the development of cerebral malaria following *Plasmodium falciparum* infection and altered the clinical outcome of this disease (McGuire et al., 1994). These observations suggest that a predisposition to increased TNFα production may be associated with the development of diabetes, systemic lupus erythematosus, and other autoimmune disorders (Picot et al., 1993, Jacob et al., 1990)

1.3.5 TNFα-mediated cytotoxicity

Although TNFα exerts pleiotropic effects on a wide variety of cell types, much attention has focused on its cytotoxic activities on malignant cell lines, and, while this may not represent the major activity of TNFα *in vivo*, this effect has certainly become a major hallmark of this cytokine. The biological activity of TNFα is usually assayed on the basis of its cytotoxic capacity; the classical cell line for testing TNFα is the fibrosarcoma L929 cell or the WEHI 164 cl 13 cell line (Ruff and Gifford, 1981, Espevik and Nissen-Meyer, 1986). Cells can die in at least two ways, either by apoptosis or by necrosis/lysis. In the case of these cell lines, TNFα leads to rapid lysis. To measure the titre of TNFα in solution, dilutions are added to cell monolayers in microtitre plates and after a few days the surviving cells which escaped lysis are quantified colorimetrically (Tada et al., 1986). There is likewise much research interest in the signalling mechanisms regulating ability of TNFα along

with other nerve growth factor (NGF)/TNF receptor family ligands (section 1.3.8) to induce apoptosis in a variety of cells in particular T-lymphocytes (Zheng et al., 1995) and HL-60 cells (Obeid et al., 1993).

1.3.6 TNF receptors

The wide range of TNFα activities is explained by the presence of TNF receptors (TNFRs) on almost all nucleated cell types. Two distinct receptor subtypes for TNFα with molecular masses of 55 kD (TNFR55) and 75 kD (TNFR75) have been identified (Hohmann et al., 1990, Brockhaus et al., 1990), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher et al., 1990, Schall et al., 1990, Smith et al., 1990, Lewis et al., 1991, Goodwin et al., 1991). The K_d values of TNFα for TNFR55 and TNFR75 are approximately 0.5 nM and 0.1 nM respectively (Loetscher et al., 1990, Schall et al., 1990, Smith et al., 1990). Of interest, human TNFα only reacts with the murine TNFR55 and not the TNFR75. TNFR55 is found on almost all cell types whereas TNFR75 is often more abundant on cells of haematopoietic lineage (Hohmann et al., 1989, Brockhaus et al., 1990, Porteu et al., 1991) and is also expressed on endothelium (Hohmann et al., 1990, Shalaby et al., 1990, Mackay et al., 1993). The extracellular domains of the two human TNFRs are 28% identical and have no more homology to each other than the other members of the NGF/TNF receptor superfamily (section 1.3.7). Likewise, there is no significant homology between the intracellular domains of the two TNFRs, indicating that these receptors almost

certainly employ different signalling mechanisms (Lewis et al., 1991). TNFR55 contains a classical 'death-domain' sequence (Tartaglia et al., 1993a) and has been reported to be the sole mediator of the TNFα-mediated death signal in most non-haematopoietic cell types (section 1.3.7). Despite this, the TNFR75, which lacks such a death domain sequence, has been proposed to play the key role in mediating TNFα-induced cytotoxicity in murine $CD8^+$ peripheral T-cells (Zheng et al., 1995).

TNFα activates the TNFRs by inducing receptor aggregation mediated through the trimeric nature of the ligand; after binding the TNFα-receptor complex is internalized and degraded, as there is no recycling. The time course of events after TNFα binds with its receptor have been followed using gold-particle labeled TNFα (Mosselmans et al., 1988). The TNFα-TNFR complexes are internalized via clatherin-coated pits (maximum reached at 5 min), move to endosomes (maximum at 15 min), and then to multivesicle bodies (maximum at 30 min), and finally end up in the secondary lysosomes, where they are degraded. The ability of TNFR-specific agonistic antibodies to mimic TNFα activities is evidence that the sole function of TNFα is clustering of its receptors and that no additional activities of this cytokine are required after the ligand-receptor complex is internalized (Espevik et al., 1990).

Both TNFR55 and TNFR75 have now been targeted and deleted in 'gene knockout' mice models; genetically engineered mice lacking the TNFR55 receptor are moderately resistant to the lethal effects of LPS but highly susceptible to infection by *Listeria monocytogenes* (Pfeffer et al., 1993, Rothe et al., 1993) while mice lacking

the TNFR75 are moderately resistant to the lethal effect of TNFα itself and to dermal necrosis evoked by repeated intradermal TNFα injections (Erickson et al., 1994). Animals lacking both TNFR55 and TNFR75 genes have the sum of these phenotypic effects but no gross developmental defects, thus consistent with the distinct structures of their cytoplasmic domains, the two TNFRs appear to fulfill different *in vivo* functions. Of interest, deletion of both the TNFα and LTα genes, which eliminates the only ligands known to interact with the two receptors, does not yield the same phenotype as deletion of both receptors. On the contrary, mice in which the LTα gene has been deleted, either alone or in combination with the TNFα gene, have no lymph nodes and Peyer's patches and no splenic white pulp with the thymus grossly preserved (De Togni et al., 1994).

The absolute number of TNFRs on human neutrophils is rather low, falling within the range of 500-6000 (Shalaby et al., 1987, Larrick et al., 1987, Schleiffenbaum and Fehr, 1990, Porteu and Nathan, 1990). Both TNFR55 and TNFR75 can be proteolytically cleaved from the cell surface and thus form soluble TNFα binding proteins (TNF-BPs), designated TNFR55-BP and TNFR75-BP, which retain their ability to bind TNFα with high affinity (Olsson et al., 1989, Seckinger et al., 1989, Engelmann et al., 1989). Several studies have now detected soluble binding proteins in the urine from febrile patients (Seckinger et al., 1988), in serum and urine from patients with renal insufficiency (Peetre et al., 1988), in normal urine (Engelmann et al., 1989), and in the serum ultra-filtrates from patients with advanced cancer (Gatanaga et al., 1990).

These TNF-BPs could be assayed on the basis of their interference with the cytolytic action of TNFα on target cells.

Neutrophils release TNF-BPs in response to both physiological and pharmacological stimuli including LPS, fMLP, the calcium ionophore A23187 and PMA (Porteau and Nathan, 1990, Lantz et al., 1994, van der Poll et al., 1995), while TNFα itself appears to regulate the expression of its receptors by inducing selective shedding of TNFR75 and internalization of TNFR55 (Porteu and Hieblot, 1994). In addition, adherence of neutrophils to a biological surface alone, without any additional stimuli, has been demonstrated to be sufficient to induce release of TNFR55-BP and TNFR75-BP from these cells (Lantz et al., 1994).

The release of TNF-BPs from neutrophils is accompanied by a decrease in TNFα binding to the cell surface (Porteau and Nathan, 1990) which correlates to a decrease in neutrophil responses to TNFα (Schleiffenbaum and Fehr, 1990) suggesting that TNF-BP release may represent a deactivation mechanism whereby the organism protects itself against an over-stimulation of these cells (Porteau and Nathan, 1990, Schleiffenbaum and Fehr, 1990).

The major function of the TNF-BPs *in vivo* appears to be to modulate the availability of biologically active TNFα; there is inhibition by competitive binding of the ligand, but such an interaction may also stabilize TNFα in a complex with TNF-BPs thereby acting as a reservoir of free and biologically active TNFα (Aderka et al., 1992).

1.3.7 NGF/TNF receptor superfamily

TNFR55 and TNFR75 are members of a superfamily of receptors referred to as the (low-affinity) NGF/TNF receptor family which are characterized by functional trimerization and the presence of one to six cysteine-rich repeats of approximately 40 amino acids in the extracellular domain that provide the motif for binding to shared structures in the ligands (Mallet and Barclay, 1991, Smith et al., 1994) as illustrated in figure 1.3.7. These receptors are widely distributed and serve co-stimulatory and/or apoptosis-inducing functions, primarily in the immune system, but also in many somatic tissues. The induction of apoptosis is most pronounced in those receptors that contain a conserved sequence in the cytoplasmic region referred to as the death domain (Tartaglia et al., 1993a, Nagata and Goldstein, 1995, Chinnaiyan et al., 1996b, Kitson et al., 1996, Marsters et al., 1996, Bodmer et al., 1997), a homologous region that allows physical association with a number of effector molecules via structurally similar death domain homology motifs.

Members of the low affinity-NGF/TNF family with intracellular death domains are TNFR55 (Loetscher et al., 1990, Smith et al., 1990), Fas/Apo-1/CD95 (Itoh et al., 1991, Oehm et al., 1992) and the very recently identified receptor WSL-1/TRAMP/Apo-3/DR3 (death domain containing receptor 3) which is closely related to TNFR55 but whose ligand remains uncertain (Chinnaiyan et al., 1996, Kitson et al., 1996, Marsters et al., 1996, Bodmer et al., 1997).

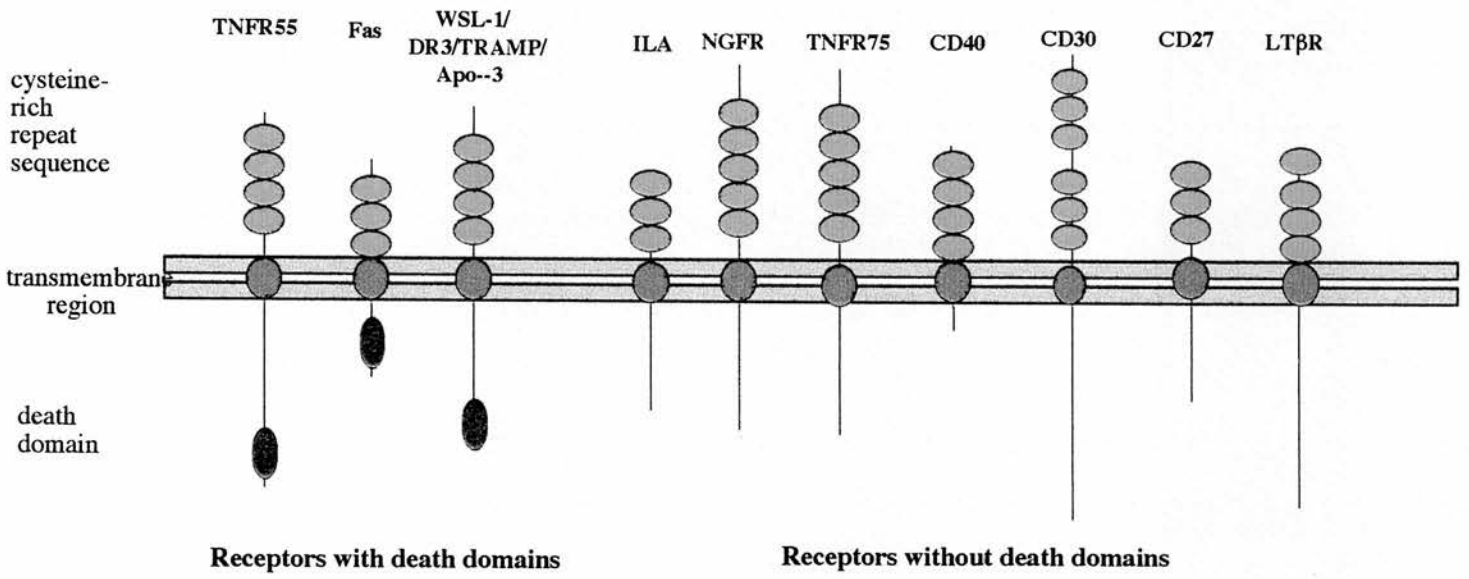


Figure 1.3.7 The NGF/TNF receptor superfamily

Although several other members of the NGF/TNF receptor superfamily lack intracellular death domains and conventionally provide a stimulatory or co-stimulatory signal for growth/proliferation, growing evidence suggests that TNFR75, CD40, and the LTβ receptor, which binds the complex between LTα and LTβ, can also trigger cell death (Bigda et al., 1994, Rabizadeh and Bredesen, 1994, Hess and Englemann, 1996, Zheng et al., 1995, VanArsdale et al., 1997). Similarly, death domain containing receptors can also provide stimulatory or co-stimulatory signals under alternative conditions that relate to the position of the cell in the cell cycle or, depending on the cell type, its activation state. Likewise, excessive signalling through some of these receptors can induce severe inflammatory reactions including tissue injury and shock. Mutations of the genes coding certain of these receptors or their respective ligands can cause characteristic disturbances of lymphocytes, derangement of the immune system or autoimmune disease.

1.3.8 NGF/TNF receptor family ligands

All of the ligands of the NGF/TNF receptor family recognize their receptors through a shared structure composed of anti-parallel β-strands arranged to form a 'jelly-roll β-sandwich'. The structural similarity of the ligands are reflected in their similar mechanisms of receptor recognition and activation. All members of the TNFα ligand family occur and act in homotrimeric forms (with the exception of the heterotrimeric LTβ which exists as a single LTα and two LTβ subunits) stabilized primarily by hydrophobic interactions, allowing each trimeric molecule to bind and aggregate

multiple receptors leading to a spatially close transmembrane cluster of cytoplasmic domains; this is thought to provide an intracellular binding site(s) for proteins that serve as the effector molecules that activate the corresponding signalling pathways. Indeed, all NGF/TNF family members are activated as a result of juxtaposition of several receptors and can thus be artificially triggered by cross-linking with antibodies. Of note, while the NGF receptor protein was actually the first family member to be identified (Johnson et al., 1986), its low-affinity dimeric ligand is structurally unrelated to the other ligands and is therefore not included as a member of the ligand superfamily.

The TNF family of ligands act not only as soluble molecules, but also when still associated with the surface of their producing cells. Most of these ligands are produced as type II transmembrane cell surface proteins with the C-terminus on the outer cell surface, a single transmembrane domain, and a short cytoplasmic tail; soluble forms are derived proteolytically. Recently, it was shown that LTα is an exception; although the homotrimer lacks a transmembrane domain and hence is exclusively secreted, the transmembrane domain of LTβ serves to anchor a LTα subunit to the cell surface in the form of a LTαβ complex (Browning et al., 1991, Ware et al., 1992). There is no evidence to suggest that the LTαβ complex is secreted. This suggests that the mode of signal transmission will depend upon cell-to-cell contact and the effects of the ligand will be restricted to the individual target cell in intimate contact with the ligand-producing cell. The ligands for the NGF/TNFR family which are currently known are summarized in table 1.3.8.

RECEPTOR	LIGAND
Fas/APO-1 (CD95)	Fas-L (Suda et al., 1993)
TNFR55 TNFR75	mTNFα sTNFα LTα
LTβR (Crowe et al., 1994)	LTα/β-heterotrimers
CD40 (Stamenkovie et al., 1989)	CD40L gp39 (Armitage et al., 1992)
CD30 (Durkop et al., 1992)	CD30L (see Gruss et al., 1994)
CD27 (Camerini et al., 1991)	CD27L (Goodwin et al., 1993a)
ILA/4-1BB (Kwon and Weissman, 1989, Schwartz et al., 1993)	4-1BBL (Goodwin et al., 1993b)
WSL-1/TRAMP/DR3/Apo-3	TRAIL? (Wiley et al., 1995)

Table 1.3.8 The NGF/TNF receptor family ligands

(mTNFα, membrane-associated TNFα; sTNFα, soluble TNFα; ILA, inducible lymphocyte activator; TRAMP, tyrosine-rich acidic matrix protein; DR3, death domain-containing receptor-3; TRAIL, TNF-related apoptosis-inducing ligand).

1.3.9 Signalling pathways involved in TNFα-mediated apoptosis

Exposure of most cells to TNFα results in activation of two transcription factors, AP-1 (Brenner et al., 1989) and NFκB (Osborn et al., 1989) which mediate induction of

cytokine and immunoregulatory genes, as well as metalloproteinases. NFκB is comprised of a homo- or heterodimer of DNA-binding proteins related to the proto-oncogene c-Rel and in most cells exists in a latent (inactive) state in the cytoplasm bound to inhibitory proteins (collectively called IκB) that mask its nuclear localization signal. Activation of NFκB by cytokines such as TNFα and IL-1 is mediated by induction of phosphorylation of IκB which leads to subsequent degradation by proteasomes (reviewed by Miyamoto and Verma, 1995). A cytokine-activated protein kinase complex named IKK (for 'IκB kinase') has recently been purified and cloned (DiDonato et al., 1997).

Several second messengers have been proposed to mediate the biological effects of TNFR ligation, including various phospholipid breakdown products, arachidonic acid metabolites, free radicals, and increased intracellular Ca^{2+} (reviewed by Beyaert and Fiers, 1994). However, as highlighted in this latter report, it is not clear whether these candidates are true second messengers or secondary effects of TNFR activation. Several protein kinases have been found to be rapidly activated in response to TNFα, including the as yet unidentified ceramide-activated kinase (Weigmann et al., 1994), IκB kinase (DiDonato et al., 1996), and a TNFR55-associated serine/threonine kinase (VanArsdale and Ware, 1994), as well as Raf-1 (Belka et al., 1995), Jun N-terminal kinases (JNKs; Minden et al., 1994), and p38/Mpk2 (Raingeaud et al., 1995). Activation of IκB kinase results in NFκB activation (Verma et al., 1995, DiDonato et al., 1996), while Raf-1, JNK and p38/Mpk2 activation contribute to induction of AP-

1 activity (Karin, 1995); however the pathways by which TNFR ligation induces activation of these protein kinases remains to be clarified.

The potential role of ceramide as a mediator of TNFα signalling has recently received much attention (figure 1.3.9A). TNFα-induced phospholipid hydrolysis can result in ceramide production (Kolesnick and Golde, 1994), and endogenous ceramide has been shown to lead to activation of NFκB (Weigmann et al., 1994) and JNK (Verheij et al., 1996) and to induce apoptosis in leukaemic cell lines (Obeid et al., 1993, Jarvis et al., 1994). It has also been proposed however that ceramide generation may be a consequence of, rather than a trigger for, TNFα-induced cell death (Beyaert and Fiers, 1994).

The identification of protein molecules that are recruited to TNFR55 and TNFR75 following ligand-induced trimerization (Rothe et al., 1994, 1995a, Hsu et al., 1995) represented a major advance in understanding the early events in TNFα signalling (figure 1.3.9B). To date, much of the knowledge of these proteins and their function has been gained indirectly, for example by two-hybrid analysis of their interactive properties in transfected yeast, or by assessment of their interactions when expressed in transfected cells at supra-physiological levels and therefore the physiological relevance of these observations must therefore be carefully evaluated.

Activation of TNFR55 leads to recruitment of the TNFR55-associated death domain protein (TRADD; Hsu et al., 1995, Hsu et al., 1996a), whereas occupancy of

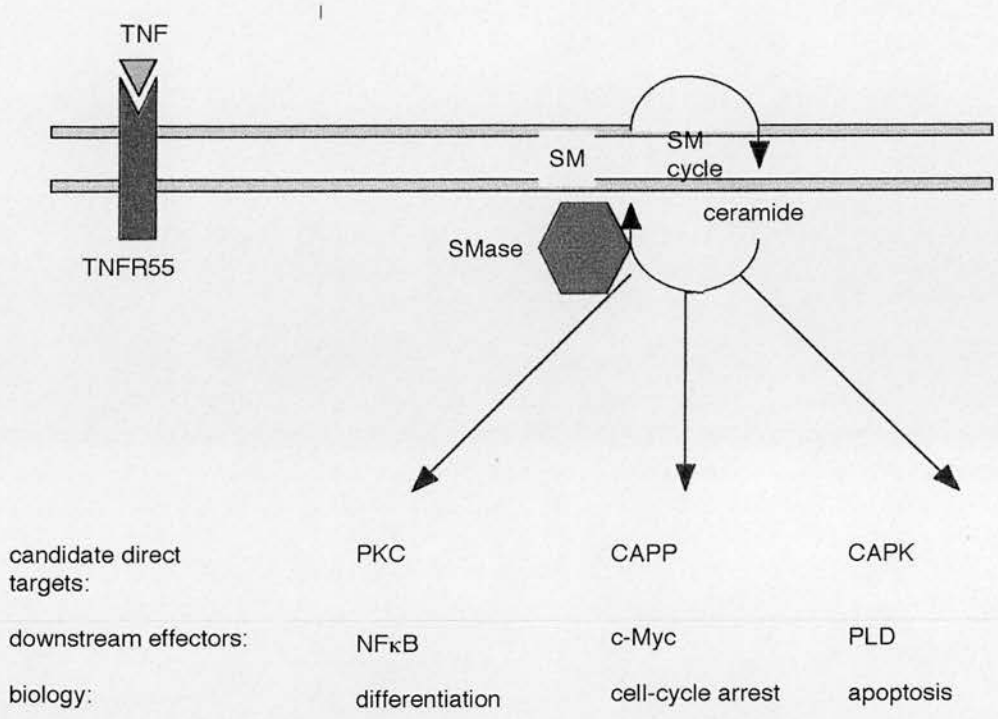


Figure 1.3.9A Proposed scheme for the role of the sphingomyelin (SM) cycle in signal transduction and the role of ceramide as a candidate second messenger.

Activation of TNFR55 results in activation of sphingomyelinase (SMase) to give rise to hydrolysis of membrane sphingomyelin (SM) and the generation of membrane ceramide. Ceramide, in turn, appears to modulate the activity of several targets, including protein kinase C isozyme ζ (PKCζ), ceramide-activated protein phosphatase (CAPP) and ceramide-activated protein kinase (CAPK). These (or other targets) then couple the action of ceramide to intracellular events, such as the activation of NFκB, activation of the retinoblastoma gene product (Rb), regulation of c-Myc expression, induction of cycloxygenase (Cox) and inhibition of phospholipase D (PLD). Importantly, the ultimate effect of generating ceramide appears to be closely related to the induction of differentiation, cell-cycle arrest, or apoptosis. These distinct biological programs appear to be cell-type dependent and are modulated by the activation of other signal transduction pathways such as the 1,2-diacylglycerol (DAG)/PKC pathway. (Adapted from Pushkavera et al., 1995).

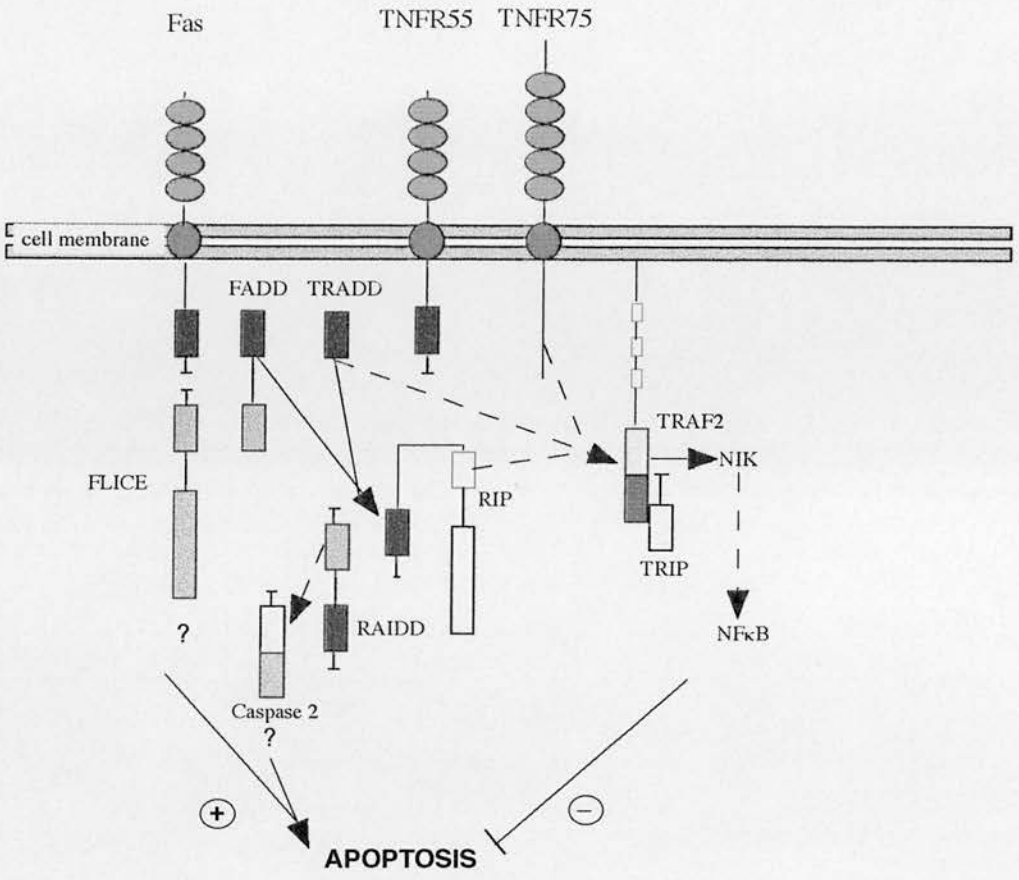


Figure 3.1.9B Schematic illustration of protein molecules recruited following ligand-induced trimerization of TNFR55, TNFR75 or Fas

Induction of apoptosis occurs by recruitment of caspase 8, and possibly also caspase 2, to the receptors through protein-protein interactions that involve homophilic binding of death domain and caspase pro-domain motifs. Cellular resistance to TNFα-stimulated cytotoxicity involves synthesis of protective proteins via the transcription factor NFκB. Activation of NFκB involves the adapter proteins TRAF and the serine/threonine kinase NIK. Motifs indicated in the figure are: the cysteine-rich extracellular-domain motif that defines the NGF/TNF superfamily members (yellow); the death domain (blue); the death effector domain, or 'MORT' domain (the caspase 8 pro-domain motif; purple); the ICE/CED3 protease sequence motif (green); TRAF2 carboxyl terminus (light pink); TRAF2 amino terminus (dark pink); serine/threonine kinase motif (pink outline). (Adapted from Wallach, 1996).

TNFR75 results in the recruitment of TNFR-associated proteins 1 and 2 (TRAF1 and TRAF2; Rothe et al., 1994). Recently TRADD was demonstrated to interact directly with TRAF2 and Fas-associated protein with death domain (FADD; Hsu et al., 1996a). The recruitment of TRAF2 to TNFR55 and TNFR75 may explain why both receptor subtypes can signal overlapping responses despite a lack of homology in their cytoplasmic domains. Indeed, TRAF2 appears to mediate both TNFR55 and TNFR75-induced activation of NFκB as well as that related to CD40 receptor stimulation (Rothe et al., 1995b, Hsu et al., 1996a).

FADD, which is also referred to as MORT1 (for 'mediator of receptor-induced cytotoxicity-1'), was originally identified as a protein which interacts with the cytoplasmic domain of Fas (Boldin et al., 1995, Chinnaiyan et al., 1995) and is structurally related to the cytoplasmic domain of TNFR55 (Itoh and Nagata, 1993, Tartaglia et al., 1993a and b). The death domain present in the cytoplasmic regions of both these receptors mediates protein-protein interactions with other death-domain containing proteins such as TRADD and FADD. Another death domain protein is the serine/threonine protein kinase RIP (for 'receptor-interacting protein'; Stanger et al., 1995, Hsu et al., 1996b) which was again originally identified by its interaction with Fas (Stranger et al., 1995) and has recently been shown to be recruited to the TNFR55 signalling complex via TRADD and to participate in NFκB activation (Hsu et al., 1996b). Studies using dominant-negative mutants have suggested that TRADD is required for TNFR55-mediated apoptosis (Hsu et al., 1996a and b) and

that expression of both TRADD and RIP death domains is sufficient to activate this process (Hsu et al., 1995, Hsu et al., 1996b, Stanger et al., 1995).

Recruitment of FADD also appears to be necessary for induction of apoptosis by TNFR55, however deletion analysis has revealed that, unlike with TRADD and RIP, this activity is mediated by a 'death effector domain' in the N-terminal portion of the molecule rather than its death domain (Chinnaiyan et al., 1995, Hsu et al., 1996a), which has paradoxically been shown to block TNFα-stimulated apoptosis (Hsu et al., 1996a, Chinnaiyan et al., 1996b). Recently, a new protein associated with the Fas-FADD and TNFR55-TRADD-FADD receptor complex has been identified which was initially named MACH (for 'MORT1-associating CED homologue'; Boldin et al., 1996), FLICE (for 'FADD-like interleukin-1β converting enzyme', ICE; Muzio et al., 1996) or Mch5 (Fernandes-Alnemri et al., 1996), and is now referred to as caspase-8 (Alnemri et al., 1996) and hence is a direct activator of the protease apoptotic cascade. The term 'caspase' was proposed to provide a unifying nomenclature for the continuously increasing list of members of the family of ICE-related proteases; the 'c' denotes the presence of cysteine at the active site, while the 'as-pase' denotes the principle cleavage activity adjacent to aspartate residues (asp-) and the number refers to the order of publication of the corresponding cDNAs (Alnemri et al., 1996).

Caspase-8 possess two motifs near its N-terminus that associate with the death effector domain of FADD which are linked to a C-terminal extension that encodes a

novel caspase. It is not clear whether caspase-8 activation following Fas or TNFR55 ligation is dependent on its proteolytic processing, either by its own activity or through effects of other proteases, or whether it occurs simply by interdigitation of several protease molecules following the binding of their pro-domains to FADD. There is evidence however, for processing of various other caspases subsequent to caspase-8 recruitment (Boldin et al., 1996, Muzio et al., 1996) which is most likely mediated by caspase-8 itself (Srinivasula et al., 1996). Thus, caspase-8 appears to be the first direct link between signalling events at the plasma membrane level and engagement of the apoptotic machinery.

The cellular targets for caspase-8 remain to be identified, however one possibility is that this enzyme may initiate a proteolytic cascade that leads to increased mitochondrial permeability transition. The importance of the mitochondria as a critical regulatory component in many forms of the apoptotic effector pathway has recently emerged (Zamzami et al., 1996a and b, Susin et al., 1997) as evidence accumulates to suggest that such a change in mitochondrial permeability is a critical event integrating signalling inputs from varying pro-apoptotic stimuli and co-ordinates the degradative processes that result in cell death. Mitochondria undergo permeability transition as a result of the formation of mitochondrial mega channels, (Zamzani et al., 1995), releasing both cytochrome C (Kluck et al., 1997) and AIF (apoptosis inducing factor; Susin et al., 1997). While the function of AIF remains to be firmly established, there is evidence to indicate that cytochrome C is necessary for activation of caspase-3. The release of cytochrome C from mitochondria is blocked

by the proto-oncogene product Bcl-2 which appears to associate with and stabilize the outer mitochondrial membrane, attenuating mitochondrial permeability transition.

The most recent discovery of a death adapter protein RAIDD suggested an additional possible route TNFR55 or Fas ligation-mediated caspase activation. This protein contains a C-terminal death domain that binds to the death domain of RIP and also contains an amino-terminal sequence homologous to that of the pro-domain of caspase-2 (ICH-1). RAIDD can bind caspase-2 and recruit it to TNFR55 through sequential interactions of RAIDD, RIP, TRADD and TNFR55, however the contribution of this pathway to TNFR55 and Fas-mediated cytotoxicity is still unknown (Duan and Dixit, 1997).

Exposure of cells which under normal conditions are resistant to TNFR55-mediated cytotoxicity to protein synthesis inhibitors can render them vulnerable to the cytocidal effects of this cytokine. These data have been taken to imply that TNFα may induce synthesis of a protein(s) which protects a cell from its own cytotoxic effect (Hahn et al., 1985). A major advance in understanding this cellular self-control mechanism was the discovery that deliberate activation of NFκB can protect cells against TNFα-mediated cytotoxicity and that by contrast, inhibition of NFκB activation can increase the sensitivity of TNFα-induced apoptosis (Beg and Baltimore, 1996, Wang et al., 1996, Van Antwerp et al., 1996, Liu et al., 1996). These observations point to NFκB as an important route of induction of anti-apoptotic protective proteins. Of interest, IL-1, which despite binding to a distinct

receptor, shares many of the pro-inflammatory activities of TNFα, and PKC-activating agents, both stimulate NFκB and enhance cellular resistance to TNFα-mediated cytotoxicity (Holtmann and Wallach, 1987).

A serine/threonine protein kinase, NIK (for NFκB-inducing kinase), which binds to TRAF2 and activates NFκB has been recently cloned and appears to be an essential component of the NFκB-activating cascade involving TNFα and IL-1. NIK shows high sequence homology to several kinases that signal within mitogen-activated protein kinase (MAPK) cascades, specifically those that act as MAPKKKs, and hence might function in a similar capacity to regulate NFκB. Over-expression of NIK in cells has been shown to increase their resistance to the TNFα-mediated cytotoxicity, whereas kinase-deficient mutants display increased TNFα sensitivity (Malinin et al., 1997).

Another component of the receptor-TRAF signally complex has very recently been cloned and named TRIP (for 'TRAF-interacting protein'; Lee et al., 1997). TRIP associates with the TNFR75 (or CD30) signalling complex through its interaction with TRAF proteins; when associated, TRIP inhibits the TRAF-2 mediated NFκB activation that is required for cell activation, and protection against apoptosis thus acting as a receptor-proximal regulator.

1.4 Aims

The major aim of the work carried out for this thesis was to determine and characterize the precise effect of TNFα on regulating neutrophil apoptosis *in vitro*. Having demonstrated that this cytokine acts in a unique way to enhance the rate of constitutive neutrophil apoptosis at early times (6 hr) while inhibiting apoptosis at more delayed times (>12 hr) the receptor subtype-dependency and regulation of these events were studied and the potential intracellular effector mediators explored.

Chapter 2: Materials and methods

2.1 Materials

The following reagents were obtained from Gibco Life Technologies (Paisley, Scotland, UK): Iscove's Dulbecco's modified Eagles medium, without supplement with L-glutamine (Iscoves DMEM) and culture supplements Penicillin (50 U/ml)/Streptomycin (50 U/ml).

The following reagents were obtained from the Sigma Chemical Company (Poole, Dorset, UK): sterile, endotoxin-free Dulbecco's phosphate buffered saline, (PBS), pH 7.4 and PBS with 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}, pH 7.4; dextran-500 (molecular weight 500) was dissolved in sterile 0.9% saline (6% w/v) and stored at 4°C); dimethyl sulfoxide (DMSO); fetal calf serum; trypan blue; propidium iodide; proteinase K; neutral sphingomyelinase (from Bacillus cereus, 50 U/µl in 50% glycerol/PBS, 50 mM Tris-HCl, pH 7.5); N6, 2'-O-dibutyryladenosine 3':5' monophosphate (dibutyryl-cAMP) was dissolved in Iscove's MDM at 20 nM and stored at -20°C; LPS (from E. Coli serotype OIII:B4, γ-irradiated) was dissolved in PBS at 1 mg/ml, sonicated (ultrawave sonic bath, Belmont Instruments, Glasgow, Scotland) for 10 min and stored at -20°C and individual aliquots were further sonicated on thawing, immediately prior to use; cytochrome C (prepared from horse heart) was stored at -20°C and dissolved in PBS pre-warmed to 37°C immediately prior to use; superoxide dismutase (SOD) was dissolved in PBS at 7500 U/ml and

stored at -20°C; PAF was dissolved in analar ethanol at 10 mM and stored at -80°C; wortmannin (from Penicillium fumiculosum was dissolved at 10 mM in DMSO and stored at -20°C; genestein was dissolved in DMSO at 100 mM and stored at -20°C, A23187 was dissolved in DMSO at 10 mM and stored at 4°C; LTB_4 was dissolved in ethanol at 100 mg/ml and stored at -20°C; sphingosine (from bovine brain) was dissolved at 15 ml in 95% ethanol/5% H_2O_2 and stored at -20oC; inositol hexakisphosphate ($InsP_6$, phytic acid, di-potassium salt) was dissolved immediately prior to use at 1 mM in PBS with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethane sulphonic acid, required to maintain pH 7.0-7.4); fMLP was dissolved in DMSO/PBS (5 mg fMLP in 70 µl DMSO made up to 11.35 ml with PBS) and stored at 1 mM at -20°C;

The following reagents were obtained from Calbiochem-Novabiochem Ltd., Beeston, Nottingham, UK: calyculin A was dissolved in ethanol at 1 mM and stored at -20°C; N-[2-bromocinnamyl(amino)ethyl]-5-isoquinoline sulphonamide, HCl (H-89, dihydrochloride) was dissolved in ethanol at 5 mM and stored at -20°C; PD 098059 was dissolved in DMSO at 20 mM and stored at -20°C; Ro31-8220 was dissolved in DMSO at 1 mM and stored at 4°C; tyrphostin AG 1288 was dissolved in DMSO at 10 mM and stored at -20°C; L-N^5-(1-Iminoethyl) ornithine, Hydrochloride (L-NIO, HCl) was dissolved in H_20_2 at 100mM and stored at -20°C; Ro20-1724 was dissolved at 100 mM in DMSO and stored at -20°C;

The following reagents were obtained from Genzyme Diagnostics, Kent, UK: rat $IgG2_b$ anti-human TNFR75 mAb (1 mg/ml in PBS) was stored at 4°C; rat $IgG_{2b\kappa\text{-}1\alpha}$ anti-human IL-2 receptor mAb (400 μg/ml in PBS) was stored at 4°C; GM-CSF (1000 U/ml in PBS) was stored at -70°C.

The following reagents were obtained from R&D Systems Europe Ltd, Oxon, UK: TNFα was dissolved in PBS at 10 μg/ml and stored at -80°C; IL-10 was dissolved in PBS at 1μg/ml and stored at -20°C; mouse IgG_1 anti-human TNFα neutralizing mAb was dissolved in PBS at 500 μg/ml and stored at -70°C; mouse IgG_1 anti-human TNFR55 mAb was dissolved in PBS at 500 μg/ml and stored at -20°C; goat IgG anti-human TNFR55 agonist pAb was dissolved in PBS at 1 mg/ml and stored at -20°C.

Dexamethasone was purchased from David Bull Laboratories (Warwick, UK) and stored at 4°C at a stock concentration of 8.3 mM; C_2- and C_6-ceramide (cell-permeable ceramide analogues), from Matreya, Pleasant Gap, PA, USA, were dissolved at 100 μM in ethanol and stored at -20°C; Diff-Quick stain [Solution I (Eosin G in phosphate buffer, pH 6.0); Solution II (Thiazine blue in phosphate buffer, pH 6.0] were from Baxter Healthcare Ltd., Glasgow, Scotland UK; Percoll was from Pharmacia fine chemicals, Uppsala, Sweden; SB 203580 from SmithKline Beecham, UK, was dissolved at 50 mM in DMSO and stored at -20°C.

All other chemicals were of molecular, reagent or cell culture grade and were obtained from BDH (Leicestershire, UK).

2.2 Methods

2.2.1 Neutrophil preparation

Human neutrophils were purified from the peripheral blood of healthy human volunteers according to the method of Haslett et al., (1985). Neutrophil isolation was performed at room temperature, under sterile conditions and using endotoxin-free reagents and plasticware (Falcon, Oxford, UK). Freshly drawn venous blood was collected into 50 ml polypropylene tubes, anticoagulated (4 ml 3.8% sodium citrate/36ml blood) and centrifuged (300g, 20 min). The platelet-rich plasma supernatant was aspirated and centrifuged (2500g, 20 min) for production of platelet-poor plasma (PPP) or used to prepare autologous serum in glass tubes by the addition of $CaCl_2$ (final concentration 20 μM) at 37°C. 5ml of 6% dextran (mol wt 500,000, 37°C) was added to pelleted cells from the initial centrifugation and 0.9% saline (37°C) added to increase the volume to 50 ml per tube. The tubes were mixed gently and allowed to stand for 30-40 min at room temperature until the majority of erythrocytes had sedimented. The leukocyte-rich plasma was aspirated, centrifuged (300g, 6 min), resuspended in 2 ml PPP and underlayered with 2 ml 42% followed by 2 ml 51% plasma-Percoll (freshly prepared from 90% Percoll in 0.9% saline, and PPP). The gradients were centrifuged (275g, 10 min) and polymorphonuclear cells harvested from the 42%/51% Percoll interface. Mononuclear cells sedimented at the PPP/42% Percoll interface. Purified cells were washed sequentially in 40 ml PPP, 40 ml Dulbecco's phosphate buffered saline (PBS), and 40 ml PBS with $CaCl_2$ and

$MgCl_2$ and the cell concentration adjusted following haemocytometer counts. Cell purity was assessed using May-Grünwald-Geimsa staining of methanol fixed cytocentrifuge preparations (figure 2.2.1); mononuclear cell contamination was routinely <0.1%.

The above density gradient centrifugation method does not separate neutrophilic from eosinophilic or basophilic granulocytes, however harvested polymorphonuclear cells generally consisted of <3% eosinophils, and basophils were rarely seen. Preparations containing >5% eosinophils were discarded. Cell viability was assessed by trypan blue exclusion and was routinely >99%. The typical yield for this isolation method was 300-600x10^6 polymorphonuclear cells/240 ml whole blood.

2.2.2 Neutrophil culture

Freshly isolated neutrophils were routinely suspended at a density of 5x10^6/ml in Iscove's modified Dulbecco's medium (MDM) supplemented with 10% autologous serum, 50 U/ml penicillin, and 50 U/ml streptomycin. Unless otherwise stated, 6.75x10^5 neutrophils were cultured in a final volume of 150 µl at 37°C in a humidified 5% CO_2 atmosphere in flat-bottomed 96-well Falcon flexiwell plates (Becton-Dickinson, UK) for the time periods indicated. Reagents to be examined in this assay system were diluted to 10x the final concentration required in serum-supplemented Iscove's MDM.

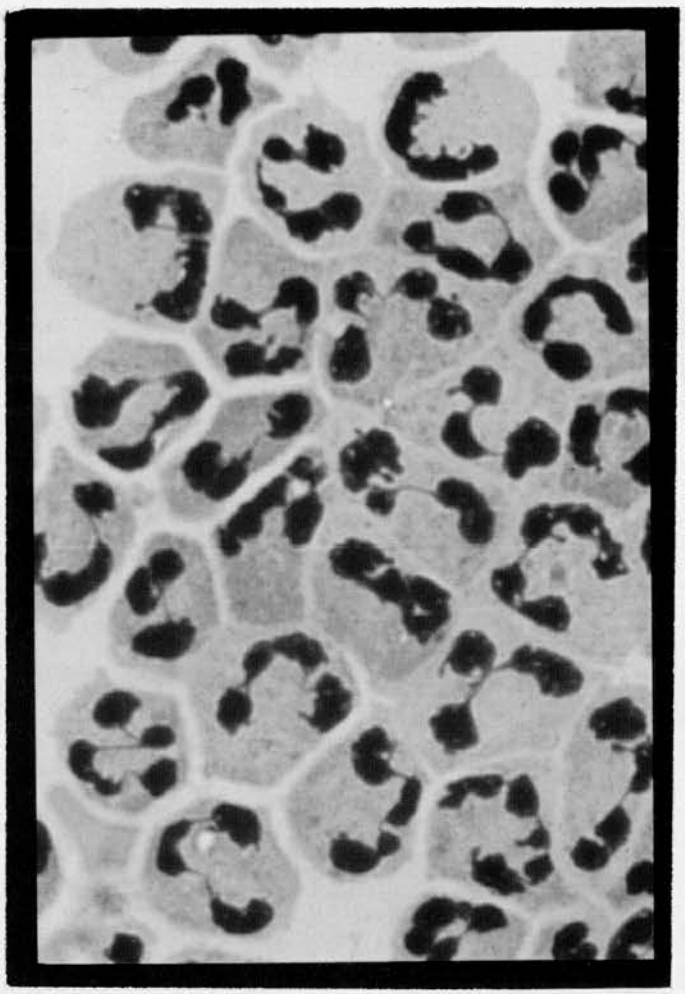


Figure 2.2.2 May/Grünwald/Giemsa-stained cytospin preparation of human neutrophils isolated on plasma/Percoll gradients

Freshly drawn, anticoagulated human blood was subjected to sedimentation with 6% dextran prior to centrifugation through discontinuous 43%/51% plasma/Percoll gradients exactly as described in 2.2. Polymorphonuclear cells were harvested from the 42%/51% interface and washed sequentially in PPP, calcium-free PBS and PBS with calcium and magnesium. 100 µl of the resulting cell suspension (approximately 10^7/ml) was spun (300g, 3 min) onto a glass slide by means of a centrifuge, and the resulting cytoprep was stained with May-Grünwald-Giemsa.

2.2.3 Assessment of neutrophil apoptosis

2.2.3a Assessment of neutrophil apoptosis by morphological criteria

Neutrophil apoptosis was assessed morphologically according to the method of Savill et al., (1989a). Cells were gently re-suspended and 100 µl (approximately $5x10^5$ neutrophils) harvested from each well, cytocentrifuged (300g, 3 min) and the resulting slide preparations air dried, fixed and stained with May-Grünwald-Giemsa. Cell viability was assessed in parallel by trypan blue exclusion. Cell morphology was examined by x100 objective oil immersion light microscopy, and apoptotic neutrophils defined as cells containing one or more darkly stained pyknotic nuclei (figure 2.2.3a). For each condition examined, slides were prepared from triplicate incubations and after coding, a total of at least 500 neutrophils were counted over a minimum of five high power fields with the observer blinded to the assay conditions.

2.2.3b Assessment of neutrophil apoptosis by propidium iodide staining

DNA fragmentation occuring during apoptosis was quantified by flow cytometric analysis of permeabilised cells stained with the intercalating fluorescent dye propidium iodide using a modification of the method of Nicoletti et al., (1991). Cells ($4x10^5$) were fixed in 100 µl ice-cold 70% ethanol at 4°C for 10 min, washed x3 (220g, 1 min) in PBS (4°C) and resuspended in 30 µl PBS and 30 µl RNAase (1 mg/ml). After gentle mixing, 60 µl of propidium iodide (100 µg/ml) was added and the cells incubated in the dark at room temperature for 15 min; cells were stored

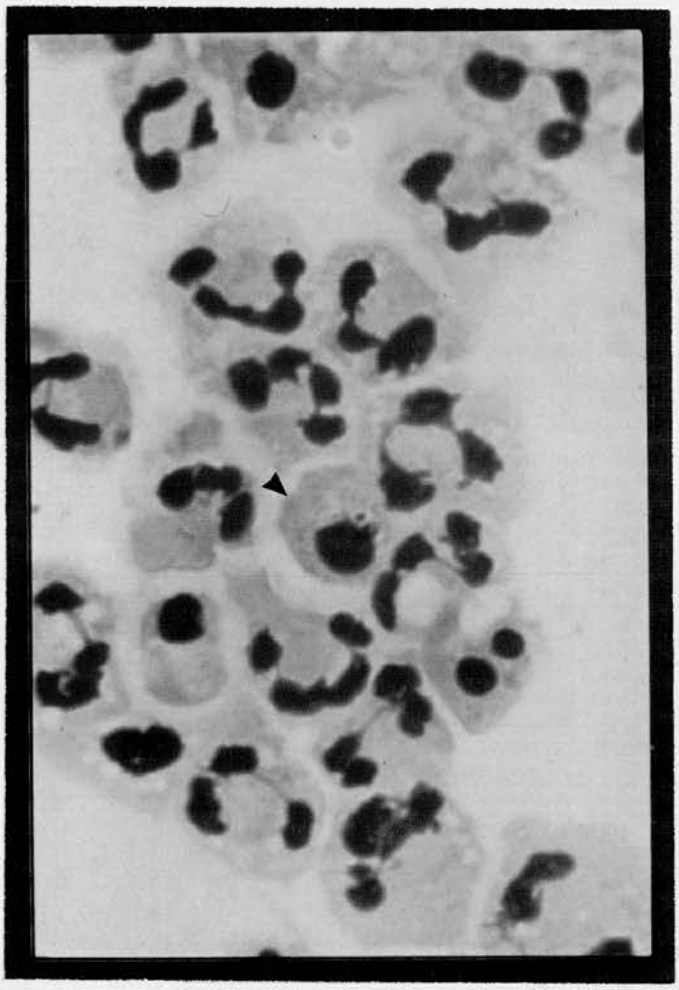


Figure 2.2.3a Assessment of neutrophil apoptosis by morphological criteria
Human neutrophils were cultured in serum-supplemented Iscove's MDM for the time period of interest; cells were resuspended and 100 μl (approximately $5x10^5$) neutrophils harvested from each well, cytocentrifuged (300g, 3 min) and the resulting slide preparations air dried, fixed and stained with May-Grunwald-Giemsa. Cell morphology was assessed by x100 oil immersion light microscopy, and apoptotic neutrophils defined as cells containing one or more darkly stained pyknotic nuclei (example arrowed).

overnight at 4°C prior to analysis using an EPICS Profile II (Coulter Electronics, Luton, UK). Mean fluorescence values from a minimum of 5000 cells were determined.

2.2.3c Assessment of neutrophil apoptosis by chromatin fragmentation assay

Neutrophil DNA cleavage was assessed using a modification of a method previously described for apoptotic lymphocytes (Trauth et al., 1989). Neutrophils were harvested at varying times from culture, centrifuged (240g, 6 min), and resuspended in 0.5 ml STE buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 1% sodium dodecyl sulphate (SDS) and 0.2 mg/ml proteinase K, and incubated overnight at 37°C. Samples were extracted three times with chloroform: isoamyl alcohol (24:1, v/v) and DNA precipitated with 2 volumes of ethanol and 0.1 volumes NaCl overnight at -20°C. Following centrifugation (13,000g, 20 min, 4°C), the remaining ethanol was allowed to evaporate at room temperature and the pellet resuspended in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) and digested with 100 μg/ml ribonuclease at 37°C for 35 min. To each sample, 0.25 volumes of loading buffer (30% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol in TBE buffer) was added and the DNA electrophoresed in a 2% agarose gel containing 75 μl ethidium bromide initially at 100V for 1 hr followed by 50V for 1 hr. In samples prepared from populations of cells containing significant numbers of apoptotic neutrophils, electrophoresis showed a typical ladder pattern which is the

biological hallmark of the endonuclease-dependent internucleosomal cleavage associated with apoptosis (Wyllie, 1980).

2.2.4 Measurement of superoxide anion generation

The release of superoxide anion was determined by means of the superoxide dismutase-inhibitable reduction of cytochrome c (see Kitchen et al., 1996b). Freshly isolated neutrophils were re-suspended at $11.1x10^6$/ml in PBS containing $CaCl_2$ and $MgCl_2$, and 90 µl aliquots transferred to 2 ml round-bottomed polypropylene Eppendorf tubes containing 10 µl TNFα (to give a final concentration of 0.05-50 ng/ml) or PBS (control), and placed in a Haake shaking water bath (100 cycles/min) at 37°C. Each reaction was performed in quadruplicate. Samples were incubated in the presence or absence of TNFα for 30 min at 37°C prior to the addition of 750 µl freshly prepared cytochrome c (final concentration 1.2 mg/ml) to all samples and 50 µl of superoxide dismutase (20 µg/ml in PBS) to one sample in each quadruplicate. After a final 15 min incubation with PBS or fMLP (100 nM), samples were placed on ice and centrifuged (10,000g, 5 min, at 4°C). The optical density of the supernatants was determined using a spectrophotometer (Pye-Unicam 8700, Unicam Ltd, Cambridge, UK) measuring peak height at 550 nm with the paired dismutase-containing supernatants as reference. Superoxide release was calculated using the extinction coefficient 21 x $10^3M^{-1}.cm^{-1}$ and expressed in nmoles released/10^6 cells.

2.2.5 ELISA assay for TNFα

Extracellular immunoreactive human TNFα was quantified using a modification of the double ligand method as previously described by Van Otteren et al., (1995). Flat-bottomed 96-well microtitre plates (Immulon 1, Dynatech) were coated with 100 µl/well mouse anti-human TNFα mAb (2.5 µg/ml in coating buffer, Dynatech) for 3 hr at 37°C and then washed with PBS (pH 7.5) containing 0.05% Tween-20 (PBS-Tween-20). Microtitre plate-associated non-specific binding was blocked with Dynatech blocking buffer (30 min, 37°C). The plates were washed four times (PBS-Tween-20) and cell-free supernatants or TNFα standards (half-log dilutions of human recombinant TNFα, from 7.8-500 pg/ml) were added in 100 µl aliquots in duplicate and incubated for 16 hr at 4°C. The plates were washed four times (PBS-Tween-20), followed by the addition of 100 µl/well rabbit polyclonal anti-human TNFα antibody (1 µg/ml in assay diluent, Dynatech) and incubated for 3 hr at room temperature. The plates were again washed, alkaline phosphatase conjugated donkey anti-rabbit IgG added, and following a further incubation of 3 hr at room temperature, p-nitrophenyl phosphate substrate added in 10% DEA buffer. The plates were then read at 410 nm in an ELISA plate reader.

2.2.6 Flow cytometric analysis of TNFR55 and TNFR75 expression in human neutrophils

Expression of TNFR55 and TNFR75 in human neutrophils was studied by indirect immunofluorescence with primary antibody directed against TNFR55 or TNFR75

and FITC-conjugated secondary antibody bound to the primary antibody detected cytofluometrically.

Neutrophils were prepared, pre-incubated for 5 min with 1 μM PAF or buffer, and then incubated ($6.75x10^5$ cells/150 μl Iscove's MDM containing 10 % autologous serum) in flexiwell plates at 37°C as detailed in 2.2.2. At the appropriate time points the cells were transferred to pre-chilled U-bottomed flexiwell plates (Becton-Dickinson, UK), washed (220g, 1.5 min at 4°C) in 100 μl ice-cold was buffer (PBS containing 0.2% BSA and 0.1% sodium azide) and resuspended in 40 μl of a saturating concentration of mouse anti-human TNFR55, TNFR75 (R&D Systems, UK), or CD2 mAb as a negative control (UCHT-1 clone, IgG_1; SAPU, Carluke, UK). After a 30 min period on ice the cells were washed twice and incubated with 40 μl FITC-conjugated goat anti-mouse immunoglobulin (Dako, Buckinghamshire, UK; diluted 1 in 40 with PBS/BSA/azide buffer). After washing, samples were analysed using an EPICS Profile II (Coulter Electronics, Luton, UK) and mean fluorescence from a minimum of 3000 cells determined.

Statistical analysis

All data are presented as mean ± SEM of the indicated number of experiments. Data were analysed by the students t-test and where appropriate by one way analysis of variance following Newman-Keuls procedure using InStat. P values less than 0.05 were considered significant.

Chapter 3: Regulation of apoptosis in human neutrophils by TNFα

3.1 Intoduction

Neutrophil apoptosis, which results in the recognition and uptake of these cells by macrophages, has been proposed as an important mechanism for the removal of neutrophils from sites of inflammation (Savill et al., 1989a and b, 1990, 1992a, Haslett et al., 1989, 1994). Experimental data obtained using human peripheral neutrophils demonstrates that these cells undergo constitutive apoptosis when aged *in vitro* and that this process is associated with maintenance of membrane integrity, hyporesponsiveness to external secretagogue signals and the capacity to be phagocytosed intact by macrophages and certain other cells with phagocytic potential (Savill et al., 1989a and b, 1990b, 1992a, Haslett et al., 1989, Whyte et al., 1993a). The speed and capacity of the macrophage phagocytic response towards apoptotic neutrophils, together with the observation that engulfment does not excite a pro-inflammatory macrophage response (Meagher et al., 1992), predicts that this process plays an important role in the safe disposal of intact but effete neutrophils from an inflamed focus. This view is supported by the recent demonstration of this process occurring *in vivo* for example in endotoxin-induced experimental lung injury (Cox et al., 1995), the neonatal respiratory distress syndrome (Grigg et al., 1991), and experimental glomerulonephritis (Savill et al., 1992b).

While the mechanism(s) regulating neutrophil survival and death are poorly understood, there is now considerable evidence to show that this process is not immutable in that the rate at which these cells undergo apoptosis, at least *in vitro*, can be altered. For example, the colony-stimulating factors G-CSF and GM-CSF, LPS, and hypoxia all increase survival in these cells by delaying apoptosis (Begley et al., 1986, Brach et al., 1992, Cox et al., 1992, Lee et al., 1993, Hannah et al., 1995). Furthermore, the inhibition of neutrophil apoptosis by LPS has been shown to be associated with prolonged functional capacity as assessed by such parameters as the ability of cells to polarize or degranulate following agonist stimulation (Lee et al., 1993). These observations imply that certain agents may serve to upregulate neutrophil function both via an early priming effect which leads to enhanced functional responsiveness to agonist stimulation and subsequently by inhibiting cell removal by delaying the onset of apoptosis.

One cytokine of particular interest in this paradigm is tumour necrosis factor-α (TNFα) since this agent is a powerful neutrophil priming agent yet has the capacity along with other NGF/TNF receptor family ligands to induce apoptosis in a number of cell types including mouse lymph node T-cells and HL60 cells (Zheng et al., 1995, Obeid et al., 1993) Despite this, a number of reports have indicated that TNFα has the opposite effect i.e. delays apoptosis in human monocytes and neutrophils (Mangan et al., 1991, Colotta et al., 1992) and prevents calcium-dependent cell death in B104 lymphoma cells (Genestier et al., 1995). The aim of the work presented in this chapter was to undertake a detailed characterization of the effect of TNFα on

neutrophil apoptosis *in vitro* and compare any such effects to the actions of other neutrophil priming agents.

3.2 Results

3.2.1 Effect of TNFα on the rate of neutrophil apoptosis *in vitro*

Co-incubation of human neutrophils with various priming agents for 20 hr either inhibited (100ng/ml LPS, 100 μM LTB_4, 500 U/ml GM-CSF) or had no effect on (1 nM fMLP, 1 μM PAF), the extent of neutrophil apoptosis (3.2.1A). Inhibition of neutrophil apoptosis at 20 hr was also observed with inositol hexakisphosphate ($InsP_6$), (figure 3.2.1A), a recently identified neutrophil priming agent (Eggleton et al., 1991, Kitchen et al., 1996a). TNFα however, although causing a significant decrease in the extent of neutrophil apoptosis observed at 20 hr caused a marked increase in morphological apoptosis at an earlier time point (8 hr) when the constitutive rate of apoptosis was still low (figure 3.2.1B). This effect was not observed with any of the other priming agents tested at this time (figure 3.2.1B).

3.2.2 Time-course for the effect of TNFα on neutrophil apoptosis

A more detailed examination of the time course of the effects of TNFα on neutrophil apoptosis is shown in figure 3.2.2. This demonstrated that TNFα induced apoptosis at 3 and 6 hr but thereafter, the rate of apoptosis in untreated cells increased rapidly so that after 12 hr incubation there was no significant difference in the extent of

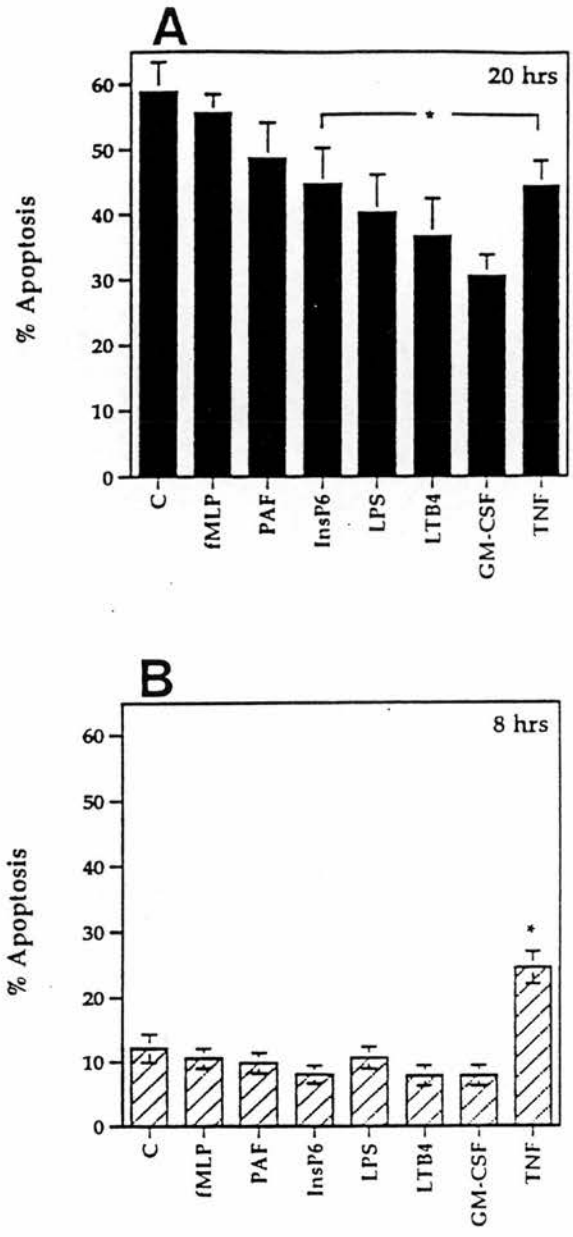


Figure 3.2.1 Effects of priming agents on neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were cultured either in serum-supplemented Iscove's MDM alone (control) or in identical medium containing: (final concentration) fMLP (1 nM), PAF (1 μM), $InsP_6$ (100 ng/ml), LTB_4 (100 nM), GM-CSF (500 U/ml), or TNFα (25 ng/ml). Neutrophils were harvested following 8 hr (B) or 20 hr (A) in culture and apoptosis assessed morphologically. Data represent mean ± SEM of 5 separate experiments each performed in triplicate. (Data were analysed by one-way analysis of variance followed by Newman-Keuls proceedure; * $p < 0.05$ compared with control values).

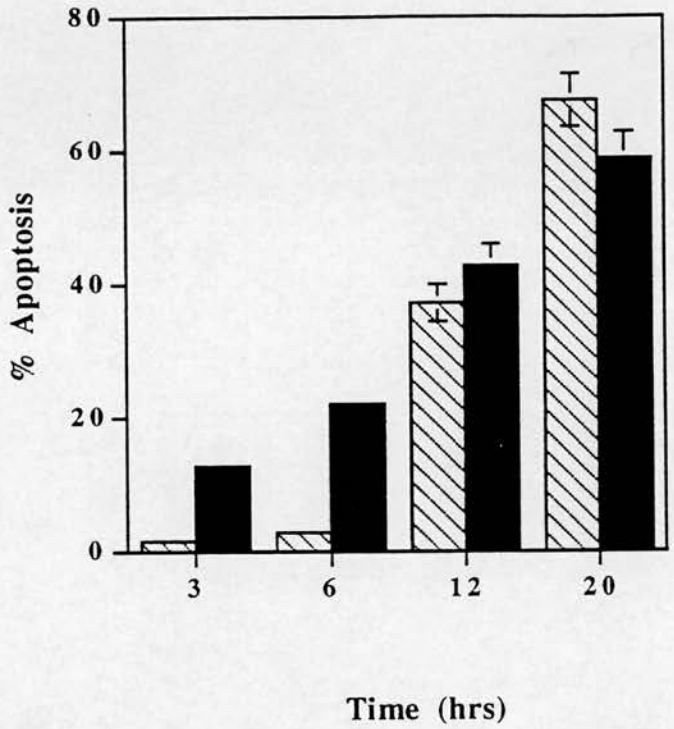


Figure 3.2.2 Time course for the effect of TNFα on apoptosis in neutrophils
Human neutrophils ($5x10^6$/ml) were incubated in serum-supplemented Iscove's MDM in the absence (hatched bars) or presence (closed bars) of 25 ng/ml TNFα. At the time periods indicated, the cells were harvested and assessed for the morphological features of apoptosis. Data represent mean ± SEM of 6 determinations from 2 independent experiments. Where not shown, SEM values are <2% of means.

apoptosis between TNFα treated and untreated neutrophils. Thereafter, the extent of apoptosis in TNFα treated cells was less than in untreated cells.

Assay of the TNFα concentrations present in cell supernatants by ELISA demonstrated no decline in the level of exogenously added TNFα over the 20 hr incubation period (table 3.2.2) and no evidence of spontaneous TNFα production from the neutrophils themselves (Xing et al., 1993; ELISA sensitivity 15.6 pg/ml).

Time (hr)	Medium + TNFα (ng/ml)	Neutrophils + TNFα (ng/ml)	Neutrophils - TNFα (ng/ml)
0	8.87 ± 0.59	10.29 ± 1.10	0*
3	8.33 ± 0.54	7.84 ± 0.62	0
6	9.18 ± 0.90	8.67 ± 0.97	0
12	8.96 ± 1.08	8.29 ± 0.68	0
20	8.69 ± 0.63	7.97 ± 0.67	0

Table 3.2.2 Effect of incubation time and neutrophil presence on the concentration of TNFα in culture supernatants

Human neutrophils ($5x10^6$/ml) or medium alone (Iscove's MDM containing 10% autologous serum) were incubated in the presence (+TNFα) or absence (-TNFα) of 12.5 ng/ml TNFα in flexiwell plates in an humidified 5% CO_2 incubator at 37°C for 0-20 hr. At the time periods indicated, the cells were centrifuged (220g, 1min), the supernatants aspirated, frozen and immunoreactive human TNFα quantified by ELISA as detailed in Materials and Methods section 2.2.5. Data represent mean ± SEM of 6 measurements performed in 2 independent experiments. (*Assay sensitivity 15.6 pg/ml).

Assessment of cell recovery (haemocytometer counts) and viability (trypan blue exclusion) at 6 and 20 hr demonstrated that TNFα did not significantly alter either of these parameters (data not shown) excluding the possibility that the increased rates of early apoptosis observed reflected either TNFα-induced adhesion of non-apoptotic cells (Schleiffenbaum and Fehr, 1990) or a TNFα-induced switch between necrosis and apoptosis as seen in human leukaemic cells treated with high concentrations of alkylating agents (Lennon et al., 1991).

3.2.3 Confirmation of TNFα-induced neutrophil apoptosis by DNA fragmentation gel analysis

DNA fragmentation is considered to be the 'biological hallmark' of apoptosis, whereby an endonuclease fragments DNA at linker sites between nucleosomes generating a population of non-random DNA fragments of defined but varying sizes. Since the DNA fragments are multimers of approximately 180 base pair nucleosomal units a characteristic ladder pattern is observed when DNA extracted from apoptotic cells is separated on agarose gels (Wyllie, 1980). Figure 3.2.3 shows that co-incubation of neutrophils with 25 ng/ml TNFα for 6 hr induces a more intense and non-random DNA fragmentation compared to non-treated cells hence confirming that the above morphological data reflects genuine apoptosis.

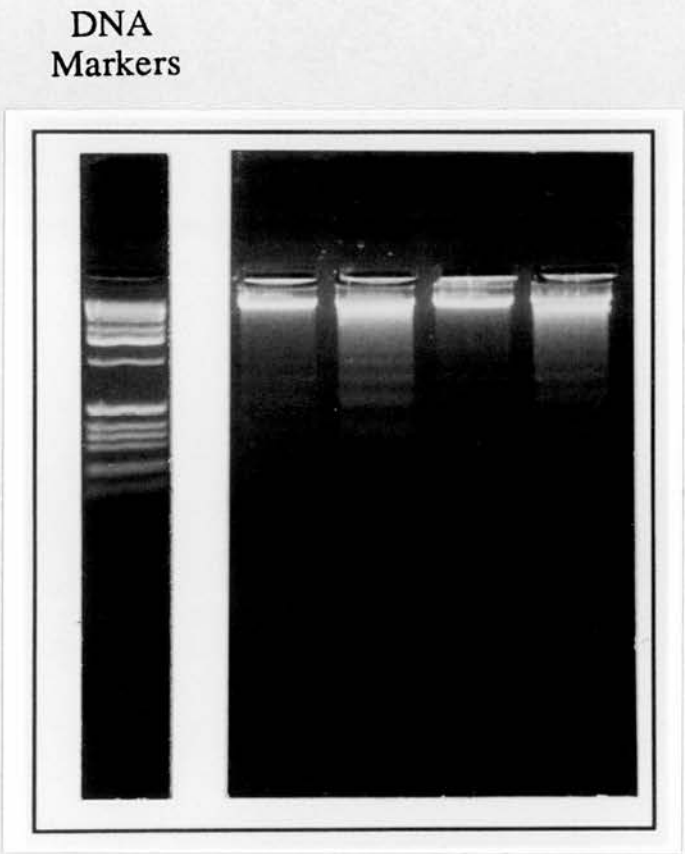


Figure 3.2.3 Effect of TNFα on DNA fragmentation in neutrophils

Human neutrophils ($5x10^6$/ml) were incubated in serum-supplemented MDM in the presence (+ lanes) or absence (- lanes) of 25 ng/ml TNFα. Cellular DNA was then extracted as detailed in Materials and Methods (2.2.3c) and separated on a 2% agarose gel. Lanes represent extracts from TNFα-treated and untreated cells from 2 separate experiments.

3.2.4 Effect of TNFα on propidium iodide staining in neutrophils

The early pro-apoptotic and late anti-apoptotic effects of TNFα on human neutrophils were further confirmed using propidium iodide DNA staining and flow cytometry. The DNA fragmentation that occurs during apoptosis results in reduced binding of propidium iodide and hence as a cell undergoes apoptosis there is decreased binding of the fluorescent dye propidium iodide to DNA, which can be analysed by flow cytometry (Nicoletti et al., 1991). Figure 3.2.4B shows the increase in the percentage of cells with hypodiploid DNA content following TNFα treatment (% apoptosis 6 hr: control 4.4 ± 0.3%, 12.5 ng/ml TNFα 18.2 ± 0.1%, $p < 0.05$, n=3). These values match exactly the extent of apoptosis assessed by morphological criteria in cells incubated in parallel under identical conditions (% apoptosis 6 hr: control 4.6 ± 0.2%, 12.5 ng/ml TNFα 17.2 ± 1.4%) (figure 3.2.4A). The late inhibitory effect of TNFα on neutrophil apoptosis (20 hr) was also confirmed using this technique (figure 3.2.4B) (% low propidium iodide stained cells 20 hr: control, 55.9 ± 0.3%, 12.5 ng/ml TNFα 46.9 ± 1.5%, $p < 0.05$, n=3).

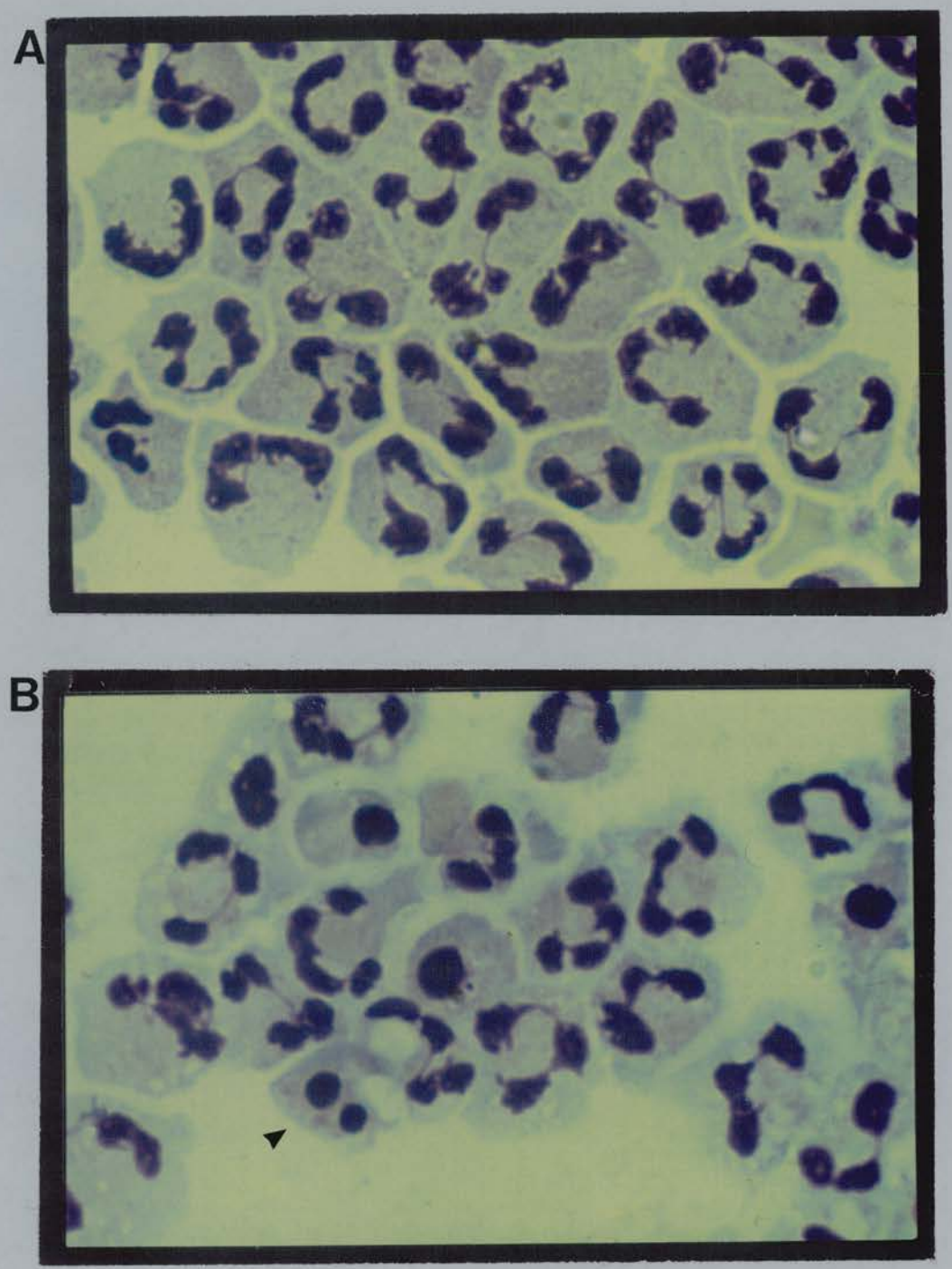


Figure 3.2.4A Effect of TNFα on neutrophil morphology

Human neutrophils ($5x10^6$/ml) were incubated in serum-supplemented Iscove's MDM in the absence (A) or presence (B) of 12.5 ng/ml TNFα. Cytospin preparations were made after 6 hr and cells were fixed, stained, and examined under 100x objective oil immersion light microscopy. Cells containing one or more darkly stained pyknotic nuclei were counted as apoptotic (example arrowed).

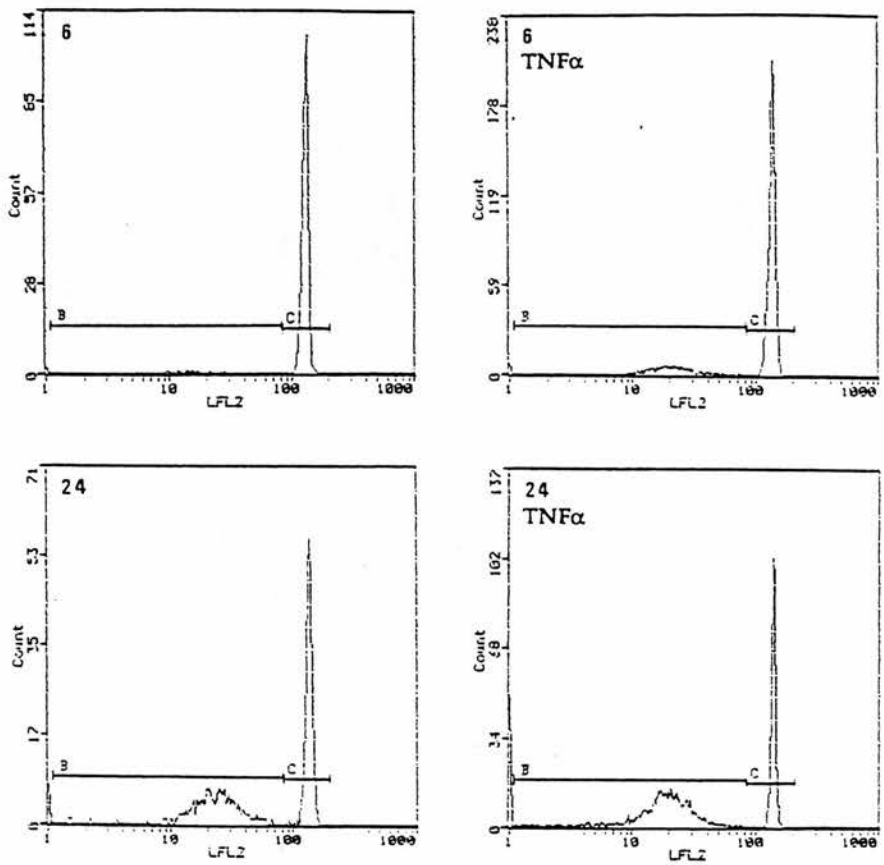


Figure 3.2.4B Effect of TNFα on propidium iodide staining in neutrophils
Human neutrophils (5x10^6/ml) were incubated in serum-supplemented MDM in the presence (right hand panels) or absence (left hand panels) of 12.5 ng/ml TNFα. Following a 6 or 20 hr incubation period cells were resuspended in ice-cold 70% ethanol, washed in PBS, and incubated with propidium iodide in the presence of RNAase prior to analysis using an EPICS Profile II. Mean fluorescence values are shown for a minimum of 5000 cells for each condition with the histograms representative of 6-9 determinations in 3 separate experiments. % Apoptosis values quoted represent % 'low' propidium iodide staining cells present.

3.2.5 Effect of TNFα neutralising antibody on TNFα-induction of apoptosis in neutrophils

To confirm the specificity of the TNFα effect i.e. to eliminate the possibility that potential contaminants present in the human recombinant TNFα preparation might be responsible for the pro-apoptotic effect observed, the effect of TNFα was examined after adsorption for 90 min at 37°C with a monoclonal TNFα neutralizing antibody. As shown in figure 3.2.5, the adsorption of TNFα with the anti-TNFα antibody eliminated the induction of apoptosis by TNFα and moreover the basal rate of apoptosis at 6 hr was not affected by the TNFα neutralizing antibody.

3.2.6 Early time-course for the pro-apoptotic effect of TNFα in neutrophils

Figure 3.2.6 demonstrates that the early pro-apoptotic effect of TNFα was rapid (observed in all experiments by 2 hr), time-dependent, and maximal in terms of the ratio of apoptotic to non-apoptotic cells by 6 hr. In view of this a 6 hr time point was selected for subsequent investigation of the pro-apoptotic effect of TNFα.

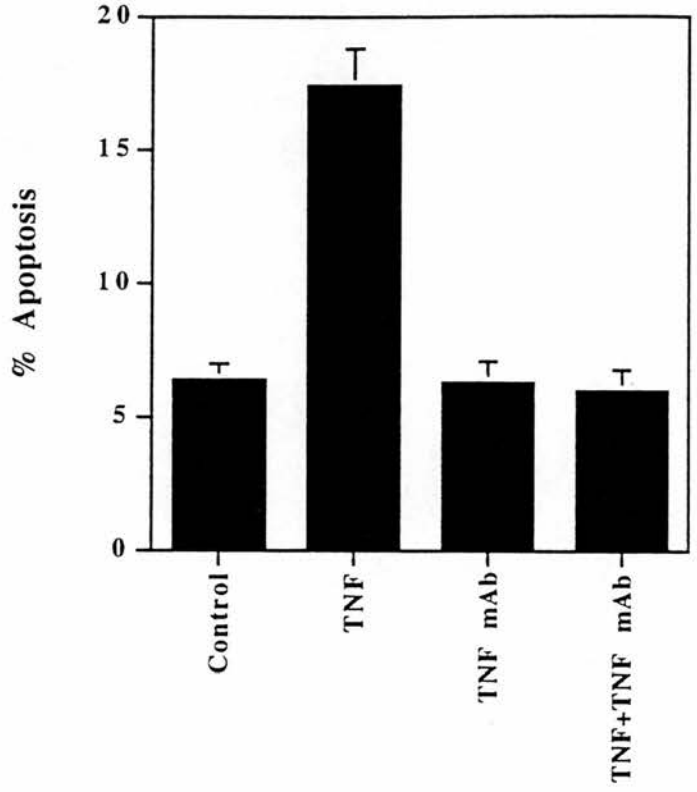


Figure 3.2.5 Effect of TNFα neutralising antibody on basal and TNFα-stimulated apoptosis in neutrophils

Human neutrophils ($5x10^6$/ml) were cultured for 6 hr either in serum-supplemented Iscove's MDM alone (control), TNFα (12.5 ng/ml), mouse IgG_1 anti-human TNFα neutralizing mAb (1 μg/ml), or an identical concentration of TNFα pre-incubated for 90 min at 37°C with the neutralising mAb (1 μg/ml). Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate.

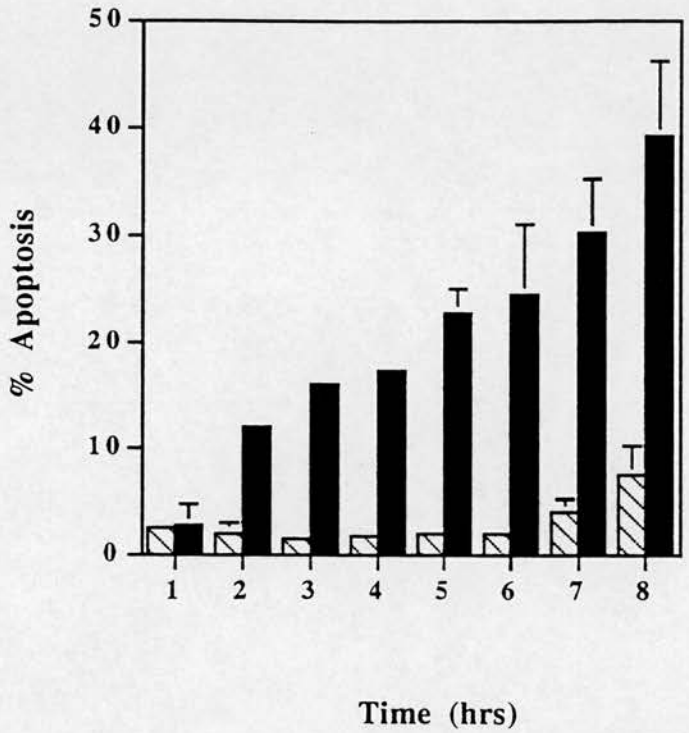


Figure 3.2.6 Early time course for the effect of TNFα on apoptosis in neutrophils

Human neutrophils ($5x10^6$/ml) were cultured in serum-supplemented Iscove's MDM in the absence (hatched bars) or presence (closed bars) of 25 ng/ml TNFα. Neutrophils were harvested hourly for 8 hr and apoptosis assessed morphologically. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate. Where not shown, SEM values are <2% of means.

3.2.7 Relationship between TNFα-induced priming, activation and apoptosis in neutrophils

To explore the concentration-dependence of the pro-apoptotic effect of TNFα and to compare the sensitivity of this response to the priming effect of TNFα, both direct TNFα–stimulated superoxide anion generation and TNFα priming of fMLP (100 nM)-stimulated superoxide anion generation were assessed in parallel. Figure 3.2.7 demonstrates that all three responses to TNFα had similar concentration-dependencies, with an EC_{50} value of 2.8 ng/ml for TNFα-induced apoptosis, however, in view of the fact that the concentration-response curves for TNFα-induced priming and direct stimulation of superoxide anion generation did not reach a plateau, it is difficult to accurately assess the potency (i.e. EC_{50}) of TNFα for these responses. From this data, a just maximal concentration of TNFα (12.5 ng/ml) was selected for use in all subsequent apoptosis studies.

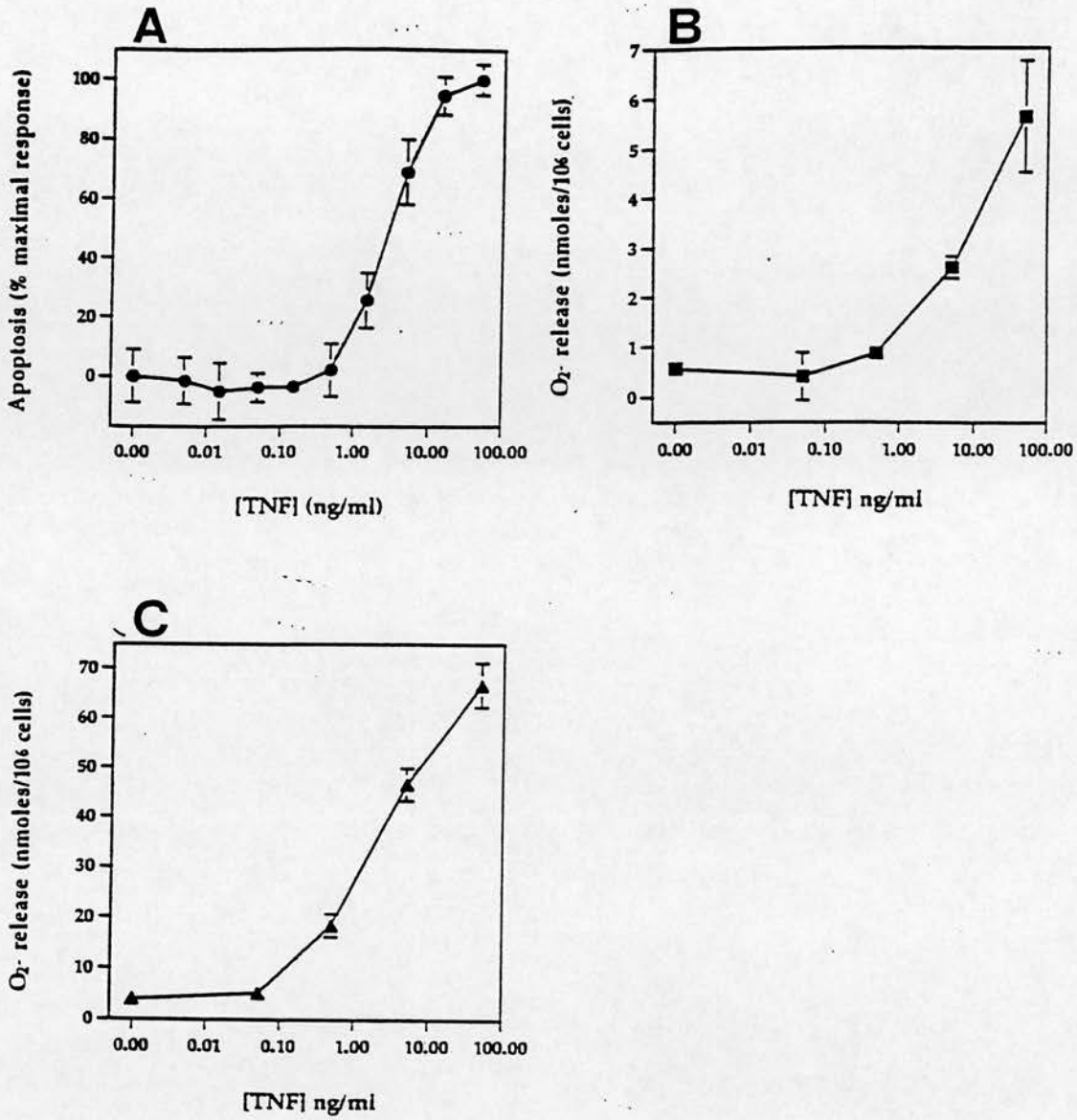


Figure 3.2.7 Concentration-response relationships for TNFα-induced apoptosis, priming and direct superoxide anion generation in neutrophils

Human neutrophils were incubated at $5x10^6$/ml with 0-100 ng/ml TNFα for morphological assessment of apoptosis at 6 hr (A) or at 10^7/ml with 0-100 ng/ml TNFα for 30 min prior to assessment of fMLP-stimulated (100 nM, 10 min) (C) or spontaneous superoxide generation (B) using a cytochrome C reduction assay (see Materials and Methods section 2.2.4). Data represent mean ± SEM from 3 separate experiments, each performed in triplicate. Where not shown, SEM values are <2% and fall within the symbols.

3.3 Discussion

In our initial experiments we confirmed the previous observation that many priming agents have the capacity also to inhibit the rate of constitutive apoptosis in neutrophils. Hence in our hands agents such as LPS and GM-CSF cause an approximately 50% reduction in the rate of apoptosis observed at 20 hr. Of note however, other agents which have an equivalent priming efficacy as LPS and GM-CSF e.g. PAF and low concentrations of fMLP (Condliffe et al., 1996, Kitchen et al., 1996b) do not modulate the extent of apoptosis at 6 or 20 hr. FMLP at a full activating concentration of 100 nM also had no effect on the apoptotic index (data not shown).

In contrast to these observations we have demonstrated that TNFα has an apparently unique bimodal effect on the rate of apoptosis in human neutrophils *in vitro* with the capacity to induce early apoptosis in addition to decreasing the extent of apoptosis at much later times. This may provide an explanation for the previous reports in which TNFα has been variably reported to inhibit (Colotta et al., 1992), have no effect on (Kwon et al., 1988), or induce apoptosis in human neutrophils (Takeda et al., 1993, Tsuchida et al., 1995).

Neutrophils appear to be highly resistant to agonist-induced apoptosis and even stimuli that are potent inducers of apoptosis in other cells types e.g. corticosteroid treatment, elevation of intracellular calcium and hypoxia, all inhibit rather than augment the rate of constitutive apoptosis in these cells (Meagher et al., 1996, Whyte

et al., 1993b, Hannah et al.,1995). Hence, unlike all other neutrophil priming and activating agents thus far tested, TNFα appears to be unique in its ability to induce apoptosis in these cells. The only other naturally occurring cytokine reported to induce apoptosis in human neutrophils is IL-6 (Afford et al., 1992). These data need to be interpreted with caution however as they were highly preliminary in nature and the incubations were performed under suboptimal culture conditions in the absence of serum, with the levels of apoptosis recorded at 24 hr in culture (10% v 15% in the presence of IL-6) outwith apoptosis rates observed by all other comparable studies. Furthermore, other groups have reported that IL-6 has no effect on neutrophil apoptosis (Brach et al., 1992, Takeda et al., 1993), or even inhibits this response (Colotta et al., 1992, Biffl et al., 1995). In the report by Biffl and co-workers (1995) it was proposed that the anti-apoptotic effect of IL-6 on human neutrophils observed was cell concentration-dependent and that this may explain the disparities in the literature. Hence they demonstrated that at cell densities of $10x10^6$/ml and $20x10^6$/ml, IL-6 inhibited neutrophil apoptosis, however at neutrophil densities of $5x10^6$/ml and below this cytokine had no effect on the spontaneous rate of cell death. They found no pro-apoptotic effect of IL-6 at any of the combinations of cell/IL-6 concentrations studied. Hence, TNFα appears to have an unique ability not shared by any other neutrophil priming and activating agent to induce neutrophil apoptosis.

The observation that only a proportion of neutrophils undergo apoptosis in response to TNFα is of particular interest since ELISA analysis of supernatants taken from cells incubated with TNFα for up to 20 hr revealed that there was no detectable

decline in the concentration of TNFα over the incubation period studied. Parallel experiments with cells cultured in the absence of TNFα also showed that this cytokine was not being produced within the range of ELISA detection (pg/ml) or therefore at levels from our concentration-response data which would affect the rate of apoptosis. Likewise there is no evidence to support heterogeneous TNF receptor expression in neutrophils since in our hands TNFα stimulates uniform CD18-dependent latex bead binding in nearly 100% of neutrophils (Condliffe et al., 1996) and flow cytometric analysis of TNFR75 and TNFR55 expression in these cells demonstrates very homogeneous receptor density between neutrophils. However, heterogeneous neutrophil responses to other stimuli such as LPS augmentation of fMLP-induced Ca^{2+} transients are well documented (Yee and Christou, 1988) and clearly other factors such as cell maturity may be important in dictating the susceptibility of individual cells to TNFα-stimulated apoptosis.

An alternative explanation to the finding that only 15-30% of neutrophils undergo apoptosis in response to TNFα is the ability of TNFα to trigger two opposing responses in these cells with the 'early' apoptotic response being modulated and finally overcome by a 'late' and evolving TNFα-induced survival effect; the number of cells undergoing apoptosis at any one time therefore reflecting the balance between these two events. This delayed protective effect of TNFα may be direct or indirect i.e. secondary to TNFα causing the release of a factor or factors that act in an anti-apoptotic 'autocrine' manner (e.g. via the generation of LTB_4; Ishii et al., 1992). It is also possible that as the neutrophils settle in the plates and a proportion adhere to

the flexiwell surface that the response to TNFα changes. Certainly this is a well recognized event with respect to the priming effect of TNFα where adhesion causes TNFα to act as a full secretagogue (Nathan, 1987, Richter et al., 1989, Nathan et al., 1989, Dri et al., 1991) i.e. adhesion may switch off or inhibit the capacity of TNFα to induce apoptosis and/or augment the protective effect and this possibility is explored further in Chapter 4.

TNFα has also been shown to enhance thrombospondin-mediated phagocytosis of apoptotic neutrophils by monocyte-derived macrophages (Ren and Savill, 1995). This suggests a potentially synergistic or dual mechanism whereby TNFα may enhance the removal of neutrophils from an inflamed site by accelerating apoptosis and facilitating phagocytic clearance. Of interest, it was also shown in this study that the neutrophil priming agent GM-CSF, which we have confirmed is a potent inhibitor of apoptosis in these cells, also upregulates phagocytosis of apoptotic neutrophils by monocyte-derived macrophages. Since the neutrophil is a short-lived cell it is interesting to speculate that, depending on the balance of inflammatory mediators present at an inflamed site, the potential exists for neutrophil function and longevity to be either up- or down-regulated and also that the subsequent clearance by macrophage phagocytes may also be under cytokine control.

Under pathophysiological conditions it is clear that the successful resolution of inflammation requires not only the switch-off or neutralization of the chemoattractant and secretagogue stimuli but also the prompt and complete clearance of influxed

granulocytes. Our data establishes the principle that neutrophil apoptosis can indeed be stimulated by a receptor operated process. This effect of TNFα may explain at least in part certain *in vivo* findings, for example how the administration of anti-TNFα antibodies results in delayed resolution of the neutrophilic alveolitis induced by *Legionella pneumophilia* (Skerrett et al., 1994), why pneumococcus (one of the most potent inducers of alveolar TNFα production; Simpson et al., 1994) causes a form of pneumonia that usually resolves with minimal residual organ damage (Robertson et al., 1938).

Several other more recent reports have demonstrated that human peripheral blood neutrophils can also be triggered to undergo apoptosis *in vitro* by a variety of other biological stimuli. A recent study by Liles et al., (1996) proposes an important role for the Fas/Fas-ligand system in the regulation of neutrophil cell death. Fas is 45 kD type I protein of the NGF/TNF receptor superfamily and activation by its natural ligand (FasL), a 37 kD type II protein, is crucial for death signalling in many cell types the most extensively studied of which is T cells (see Liles et al., 1996). This study demonstrated that Fas and FasL are constitutively expressed in neutrophils, unlike in T cells, which have an extremely low rate of constitutive apoptosis, and require cell activation for the expression of FasL. In the above study stimulation with anti-Fas agonistic antibodies (CH-11) induced rapid neutrophil death within 3 hr, and antagonistic anti-Fas antibodies reduced spontaneous neutrophil death by approximately 50% implying that Fas may play a key role in the regulation of constitutive neutrophil apoptosis. In addition, these studies also showed that

neutrophils release a soluble factor, of the same molecular weight as FasL, which could induce apoptosis in Fas-sensitive Jurkat cells suggesting that neutrophils may represent a biological source of this ligand, perhaps important in the paracrine and autocrine control of cell death.

Another recent study by Watson et al., (1996) demonstrated that ingestion of *Escherichia coli* by neutrophils *in vitro* resulted in subsequent cell death by apoptosis. This phagocytosis-mediated induction of cell death was dependent upon production of reactive oxygen intermediates, which was not required for the regulation of spontaneous neutrophil apoptosis, in agreement with previous work from our own laboratory (Hannah et al., 1995). Further studies by Coxon et al., (1996) showed that neutrophil apoptosis can also be induced *in vitro* following phagocytosis of complement-opsonized oil red particles. This was again reactive oxygen intermediate-dependent and was blocked by antagonistic antibodies to CD11b/CD18 which did not attenuate the constitutive rate of apoptosis of neutrophils not fed opsonized particles. These data propose a novel role for β_2 integrins in the regulation of phagocytosis-induced neutrophil apoptosis. TNFα remains, however, the only physiological neutrophil priming and/or activating agent with the ability to rapidly induce apoptosis in these cells.

In this context in many inflammatory models TNFα is recognized as being the dominant 'early wave' cytokine present at such sites and hence there may be an early 'window of opportunity' to achieve rapid granulocyte clearance at a stage where the

build up of alternative cytokines that delay this process has yet to occur. Such *in vivo* studies also indicate that the concentration of TNFα present at inflamed sites is frequently >10 ng/ml and hence confirming the likely physiological relevance of the *in vitro* TNFα apoptosis effect (Aggarwal and Puri, 1995). It is also possible that in patients with severe sepsis associated with systemic TNFα release that TNFα may induce intravascular apoptosis and contribute towards the accompanying neutropenic state. In this context recent data demonstrating variations in TNFα gene promoter region and hence TNFα generation between individuals (McGuire et al., 1994) may have a bearing on the beneficial as well as the detrimental effects of TNFα.

Chapter 4: Exogenous regulation of TNFα-induced neutrophil apoptosis

4.1 Introduction

TNFα is a potent pro-inflammatory cytokine produced mainly by activated macrophages and monocytes, but also by lymphocytes, fibroblasts, mast cells and even neutrophils themselves (Dubravek et al., 1987). TNFα is expressed as a 26 kD integral transmembrane precursor protein which is proteolytically cleaved to release a 17 kD subunit (Kriegler et al., 1988). Both the secreted 17 kDa protein and the 26 kDa pre-peptide spontaneously trimerize and form the bioactive ligand (Smith and Bagiolini, 1987). The membrane-bound form of TNFα is as active as the soluble form in terms of cytotoxicity and anti-tumour activity (Kriegler et al., 1988, Perez, 1990) and has recently been demonstrated to have a pro-apoptotic effect on WEHI 164 cells (Monasta et al., 1996).

TNFα serves to upregulate many aspects of neutrophil function including priming for enhanced responsiveness to secretagogue agonists (Klebanoff et al., 1986, Shalaby et al., 1985, Atkinson et al., 1988), promotion of adherence to endothelial cells and cells coated with extracellular matrix proteins (Gamble et al., 1985, Dri et al., 1991, Thompson and Matsushima, 1992), stimulation of phagocytosis (Shalaby et al., 1985, Klebanoff et al., 1986) and in adherent cells can trigger degranulation and respiratory

burst activity directly (Nathan et al., 1987, Richter et al., 1989, Nathan et al., 1989, Dri et al., 1991).

In the previous chapter an anti-inflammatory role for TNFα was proposed based on the demonstration of a pro-apoptotic effect of TNFα in human neutrophils. In view of recent reports demonstrating an inhibition of TNFα-stimulated human neutrophil apoptosis by LPS *in vitro*, and insensitivity of rat inflammatory neutrophils (obtained after intra-peritoneal injection with streptococcus preparation) to the cytotoxic effects of TNFα (Hachiya et al., 1995, Tsuchida et al., 1995) we were particularly keen to examine whether the pro-apoptotic effect of TNFα is influenced or regulated by other factors relevant to the inflamed site. Hence this chapter examines the importance of the functional status of the neutrophil (i.e. quiescent vs. primed), the effects of cell adherence (which dramatically alters other functional responses of neutrophils to TNFα; Nathan, 1987, Nathan et al., 1989, Richter et al., 1989, Dri et al., 1991), and the presence of other cytokines on the pro-apoptotic effect of TNFα with a view to understanding how TNFα may regulate neutrophil apoptosis *in vivo*. Since the pro-apoptotic effect of TNFα in other cell types is also highly dependent on culture conditions (Beyaert and Fiers, 1994), we were keen to explore different strategies to enhance the efficacy of the pro-apoptotic effect of TNFα and optimize our culture system for subsequent mechanistic studies.

4.2 Results

4.2.1 Inter- and intra- donor variability of TNFα-stimulated neutrophil apoptosis

In view of our observation that only a proportion of the total neutrophil pool undergo apoptosis when stimulated with TNFα *in vitro*, and recent data indicating striking inter-individual variations in TNFα synthesis due to a functional polymorphism at the promoter region of the TNFα gene, termed the TNF2 allele (McGuire et al., 1984), the relative pro-apoptotic effect of TNFα on neutrophils from different donors was compared to establish a range of responsiveness to this cytokine. Neutrophils prepared from different donors varied with respect to their susceptibility to TNFα-induced death (figure 4.2.1A), the 'apoptotic index' (difference at 6 hr between % apoptosis in TNFα-treated cells % apoptosis in control cells) ranging from 10.4% to 35.8% (median response: control 1.9%, TNFα 20.6%, n=11). To determine whether there was a consistent effect within individual donors for neutrophil responsiveness to TNFα, cells were prepared from three donors on two occasions (at least twelve weeks apart) and the rates of TNFα-stimulated apoptosis compared. Figure 4.2.1B demonstrates that the intra-donor variability for TNFα-induced neutrophil apoptosis was in fact very small with individuals showing a fairly stereotyped TNFα response across time. This data also implies that minor day-to-day variation in cell preparation does not dramatically alter the TNFα pro-apoptotic effect.

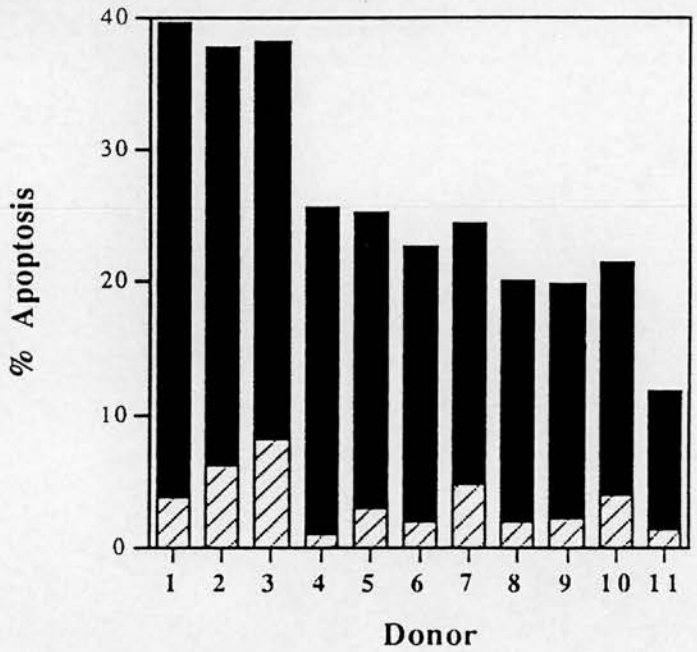


Figure 4.2.1A Inter-donor variability of TNFα-stimulated neutrophil apoptosis
Human neutrophils were purified from eleven donors and incubated ($5x10^6$/ml) in serum-supplemented Iscove's MDM in the absence (hatched bars) or presence (closed bars) of 12.5 ng/ml TNFα. Neutrophils were harvested following 6 hr in culture and assessed for morphological features of apoptosis. Data represent mean values of triplicate determinations made in eleven independent experiments. SEM are not shown for clarity but were consistently <1.6% of the mean values.

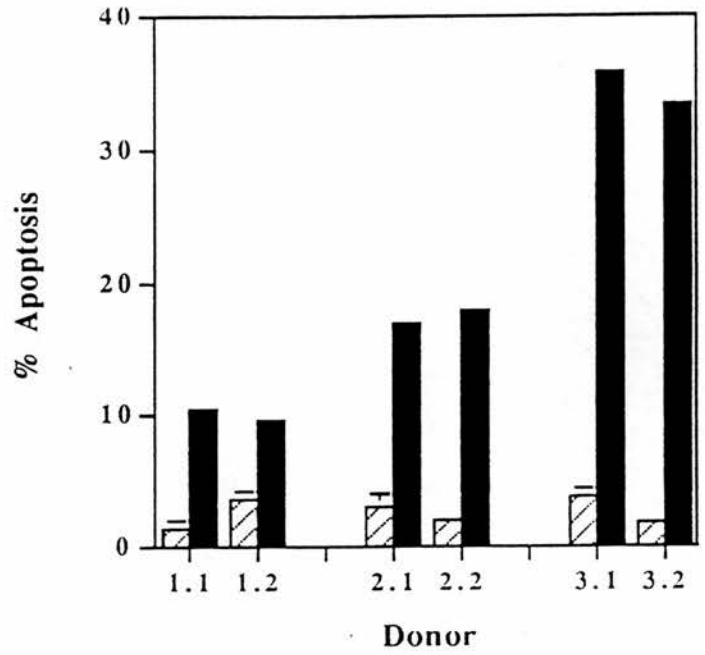


Figure 4.2.1B Intra-donor variability of TNFα-stimulated neutrophil apoptosis
Human neutrophils from three donors (1, 2 and 3) were purified on two separate occasions at least twelve weeks apart and incubated ($5x10^6$/ml) in serum-supplemented Iscove's MDM in the absence (hatched bars) or presence (closed bars) of 12.5 ng/ml TNFα. Cells were harvested following 6 hr in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM from triplicate determinations made in six independent experiments; where not shown, SEM values are <2% of means.

4.2.2 Effect of oral corticosteroid treatment on the pro-apoptotic effect of TNFα in neutrophils

In an attempt to determine whether neutrophil maturity is an important factor dictating the susceptibility of individual cells to apoptosis, we compared the pro-apoptotic effect of TNFα on neutrophils from two volunteer donors both before and during daily administration of oral glucocorticoid treatment (Prednisolone), which is known to increase bone marrow release of neutrophils into the peripheral blood (Dale et al., 1975). A dramatic increase in neutrophil responsiveness to TNFα-mediated apoptosis was observed on day four of glucocorticoid treatment (Prednisolone, 30 mg once daily) and this augmented response was sustained throughout the duration of glucocorticoid administration (figure 4.2.2). Neutrophil counts ($x10^6$/l whole blood) were monitored in parallel and a steady increase was observed from pretreatment levels of 3.48 and 3.88 to 9.26 and 7.10 respectively on day seven.

In view of previous reports demonstrating the inhibition of neutrophil apoptosis by dexamethasone *in vitro* (Cox, 1995, Meagher et al., 1996), and more recent data demonstrating partial inhibition of a TNFα-mediated neutrophil apoptosis *in vitro* by dexamethasone (Kato et al., 1995), exogenous addition of this synthetic glucocorticoid was also included in the study. As shown in figure 4.2.2, 1 μM dexamethasone partially inhibited TNFα-induced neutrophil apoptosis but this attenuation of the pro-apoptotic effect of TNFα was of similar magnitude both before and during Prednisolone administration. Hence the enhanced rates of TNFα-induced apoptosis observed in Prednisolone treated neutrophilic individuals was preserved even in the presence of dexamethasone.

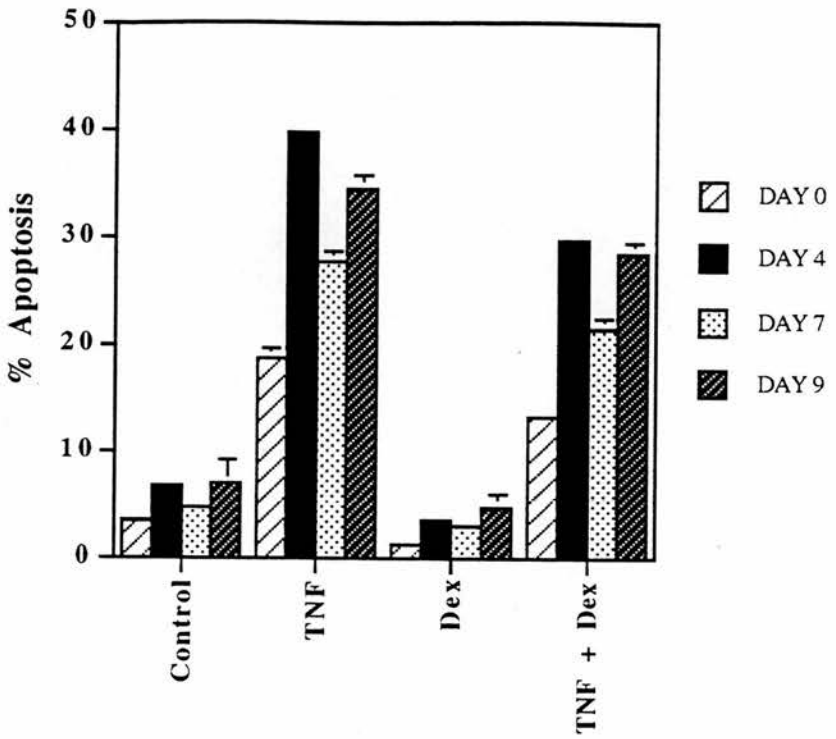


Figure 4.2.2 Effect of oral corticosteroid treatment on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils were purified from two individual donors on four separate days (denoted 0, 4, 7 and 9 to indicate days of steroid administration, 30 mg/day single dose Prednisolone, which commenced post-venipuncture on day 0. Neutrophils ($5x10^6$/ml) were incubated in serum-supplemented Iscove's MDM (control) or in identical medium containing: (final concentration) TNFα (12.5 ng/ml), dexamethasone (Dex, 1 μM), or TNFα (12.5 ng/ml) plus dexamethasone (1 μM). Neutrophils were harvested following 6 hr in culture and apoptosis assessed morphologically. Data represent mean ± SEM of 6 determinations from 2 independent experiments performed in triplicate; where not shown, SEM values are <2% of means.

4.2.3 Effect of cell concentration on the pro-apoptotic effect of TNFα in neutrophils

In view of recent data which demonstrated that the anti-apoptotic effect of IL-6 in human neutrophils was cell concentration dependent (Biffl et al., 1995), the rate of apoptosis in neutrophils cultured at different densities in the presence, and absence of TNFα was compared. Figure 4.2.3 demonstrates that the pro-apoptotic effect of TNFα was most marked at the lowest cell concentration studied ($2.5x10^6$ neutrophils/ml) and decreased as the cell concentration was increased up to $10x10^6$/ml, however no further decline in the extent of TNFα-stimulated neutrophil apoptosis was observed when the cell concentration was increased from $10x10^6$-$15x10^6$ neutrophils/ml. A cell concentration of $5x10^6$/ml was selected for use in subsequent studies as this was the optimal density for preparation and quantification of cytospins.

4.2.4 Effect of serum concentration on the pro-apoptotic effect of TNFα in neutrophils

The pro-apoptotic effect of TNFα was not affected by varying the concentration of autologous serum (up to 10% v/v), serum withdrawal, or substitution with 10% fetal calf serum (figure 4.2.4); thus 10% autologous serum was used in all subsequent studies.

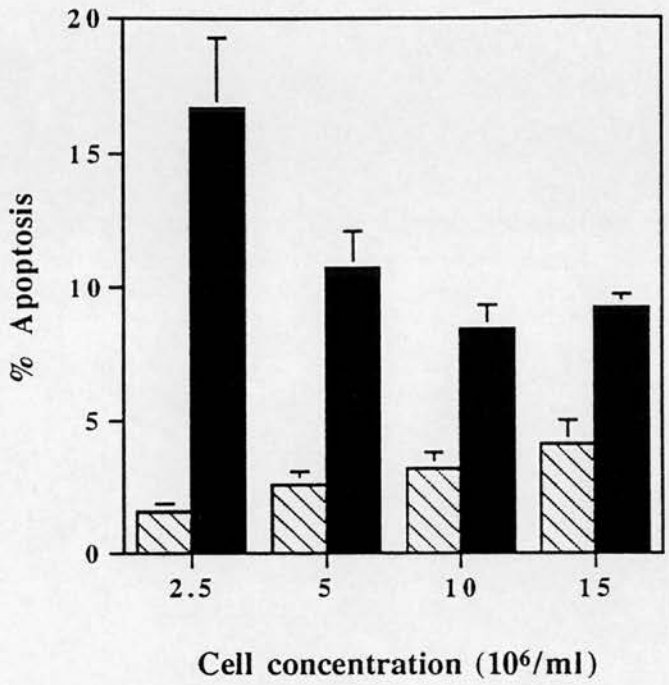


Figure 4.2.3 Effect of cell concentration on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils were incubated in serum-supplemented Iscove's MDM at densities of 2.5, 5, 10 or 15x10^6/ml in the absence (hatched bars) or presence (closed bars) of 12.5 ng/ml TNFα. Neutrophils were harvested following 6 hr in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 6 determinations from 2 independent experiments performed in triplicate.

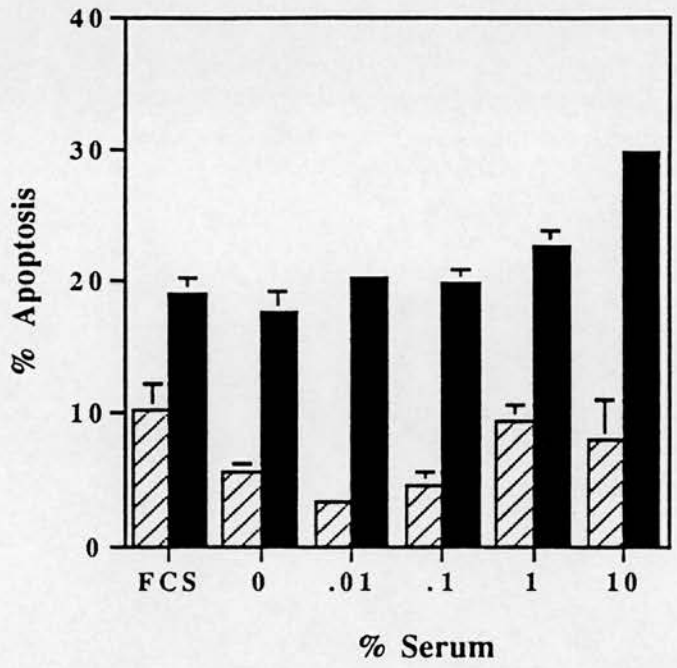


Figure 4.2.4 Effect of serum concentration on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils ($5x10^6$/ml) were cultured in Iscove's MDM alone or in identical medium containing: (final concentration) fetal calf serum (FCS, 10%), or autologous serum (0.01-10%) in the absence (hatched bars) or presence (closed bars) of 12.5 ng/ml TNFα. Neutrophils were harvested following 6 hr in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 6 determinations from 2 independent experiments. Where not shown, SEM values are <2% of means.

4.2.5 Effect of pre-incubation of cells on the pro-apoptotic effect of TNFα in neutrophils

In view of our observations that in particular experiments where pre-incubation of neutrophils was required prior to stimulation with TNFα the pro-apoptotic effect of TNFα was reduced, the relative rates of TNFα-stimulated apoptosis in cells exposed to TNFα immediately was compared to that in cells pre-incubated prior to addition of this cytokine. In these experiments the pro-apoptotic effect of TNFα was shown to be dramatically abrogated when neutrophils were pre-incubated for 30 min at 37°C in flexiwell plates (figure 4.2.5), however the cytotoxic effect was partially preserved when cells were pre-incubated in suspension for the same time period by gentle agitation in a shaking water bath in polypropylene Eppendorf tubes (figure 4.2.5) implying that adherence of neutrophils might be one of the factors contributing to this loss of the pro-apoptotic effect of TNFα.

4.2.6 Effect of delayed addition of TNFα on neutrophil apoptosis

To assess further the effect of delayed addition of TNFα, neutrophils were pre-incubated in flexiwell plates for 6 hr to allow the cells to adhere fully to the flexiwell containers prior to addition of TNFα. This pre-incubation completely abrogated the pro-apoptotic effect of TNFα (figure 4.2.6). Of note however, despite the complete loss of the early pro-apoptotic effect of TNFα when addition was delayed by 6 hr, the late inhibitory effect on apoptosis (22 hr) was preserved and even appeared of greater magnitude (figure 4.2.6).

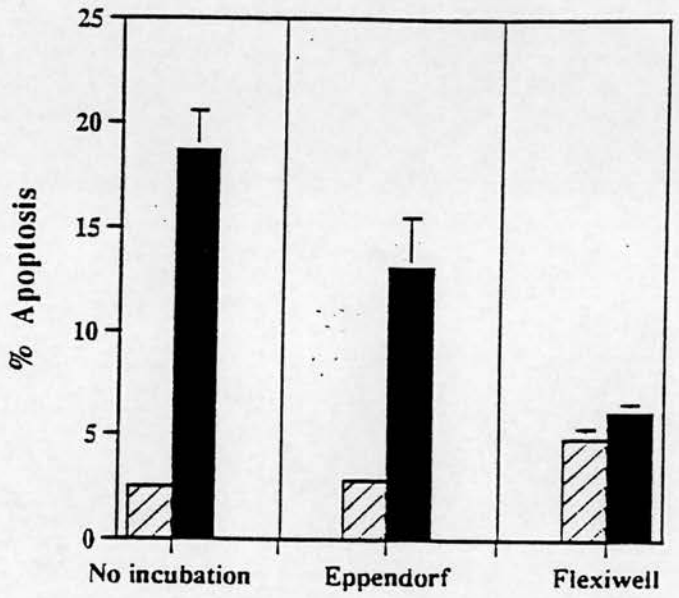


Figure 4.2.5 Effect of pre-incubation of cells on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils ($5x10^6$/ml) were cultured in serum-supplemented Iscove's MDM in the absence (hatched bars) or presence (closed bars) of 12.5 ng/ml TNFα in flexiwell plates for 6 hr prior to morphological assessment of apoptosis. Left hand panel: TNFα was added to cells at the beginning of the incubation period performed in flexiwell plates. Middle panel: neutrophils were pre-incubated in 2 ml polypropylene Eppendorf tubes in a shaking water-bath at 37°C for 30 min prior to culture in flexiwell plates in the presence or absence of TNFα. Right hand panel: neutrophils were pre-incubated in flexiwell plates for 30 min at 37°C prior to addition of TNFα. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate. Where not shown, SEM values are <2% of means.

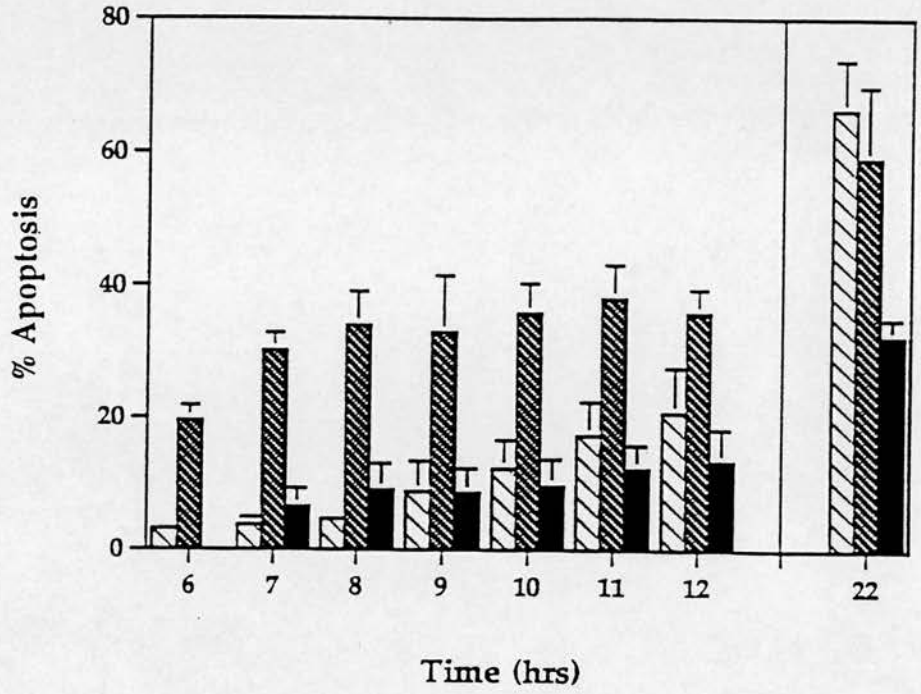


Figure 4.2.6 Effect of delayed addition of TNFα on neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were incubated in the absence (wide hatched bars), or presence of 12.5 ng/ml TNFα added either at the beginning of the incubation period (closed hatch bars) or after a delay of 6 hr (filled bars). Neutrophils were harvested at the time points indicated and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate. Where not shown, SEM values are <2% of means.

4.2.7 Effect of pre-treatment with PAF on basal and TNFα-stimulated apoptosis in neutrophils

To assess whether the induction of apoptosis by TNFα was influenced by neutrophil priming, cells were pre-incubated with 1 μM PAF for 5 min prior to the addition of TNFα. This protocol results in a dramatic upregulation (priming) of fMLP-stimulated superoxide anion release (Kitchen et al., 1996b). As shown in figure 4.2.7, PAF alone did not influence the rate of apoptosis at 6 hr but completely abolished the pro-apoptotic effect of TNFα.

4.2.8 Effect of Interleukin-10 on basal and TNFα-stimulated apoptosis in neutrophils

Incubation of neutrophils with the anti-inflammatory cytokine IL-10 (1-100 ng/ml) had no influence on either constitutive or TNFα-stimulated neutrophil apoptosis at 6 hr (figure 4.2.9). The biological activity of IL-10 was confirmed in these experiments by demonstrating its ability to block LPS-stimulated TNFα release from monocyte-derived peripheral blood macrophages as detected by ELISA (L Bruce, personal communication; Ward et al., 1997).

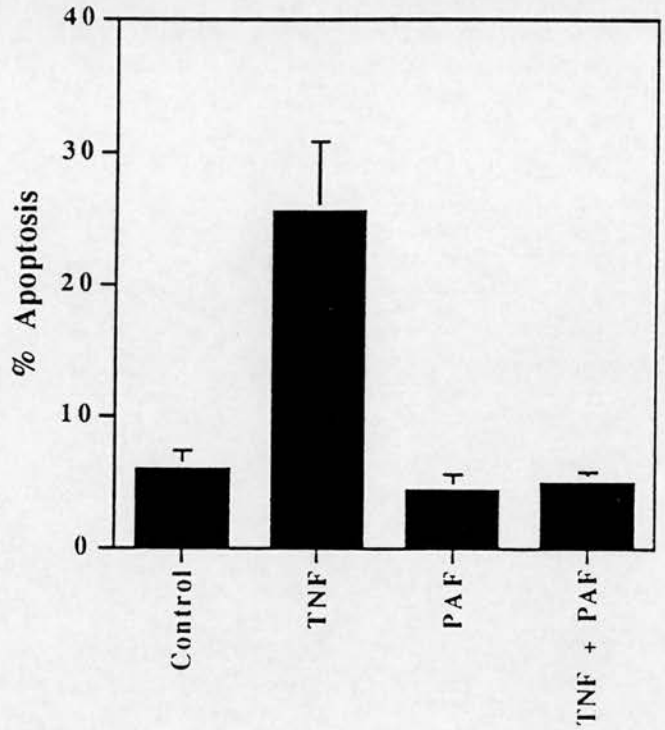


Figure 4.2.7 Effect of pre-treatment with PAF on basal and TNFα-stimulated apoptosis in neutrophils

Human neutrophils ($5x10^6$/ml) were pre-incubated in serum-supplemented Iscove's MDM in the presence or absence of PAF (1 μM) for 5 min at 37°C prior to culture for 6 hr in the presence or absence of 12.5 ng/ml TNFα. Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate.

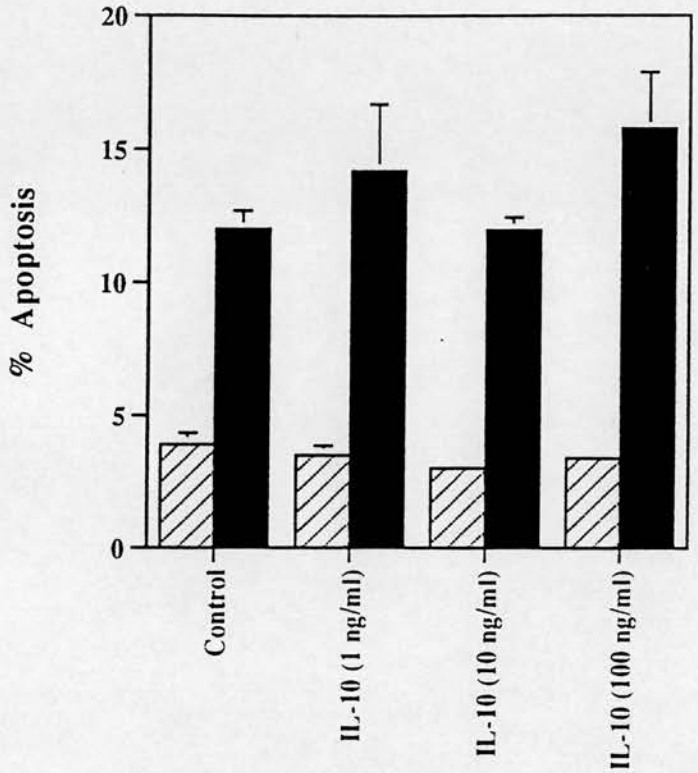


Figure 4.2.8 Effect of Interleukin-10 on TNFα-stimulated neutrophil apoptosis
Human neutrophils ($5x10^6$/ml) were incubated in serum-supplemented Iscove's MDM alone (control) or in identical medium containing interleukin-10 (IL-10, final concentration 1-100 ng/ml) in the absence (hatched bars) or presence (closed bars) of 12.5 ng/ml TNFα. Neutrophils were harvested following 6 hr in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of triplicate determinations in a single representative experiment of 4; where not shown, SEM values are <2% of means.

4.3 Discussion

In the initial studies we established that different donors display a range of responsiveness of their peripheral blood neutrophils to the pro-apoptotic effect of TNFα. Moreover, we demonstrated consistency within individual donors with regard to the pro-apoptotic effect of TNFα on isolated neutrophils. To minimize potential diurnal variations of cellular responsiveness to TNFα, donor venipuncture, subsequent neutrophil isolation and experimentation was performed for all experiments at approximately the same time of day (9 am ± 1 h). This controls for minor variations in corticosterone levels, which result in an increased release of bone marrow neutrophils (Dhabhr et al., 1994), that have been shown to inhibit neutrophil apoptosis *in vitro* (Cox, 1995, Meagher et al., 1996). It is now well recognized that inter-individual differences exist in TNFα production by LPS-stimulated human peripheral blood mononuclear cells (Molvig et al., 1988) and a recent study by McGuire and colleagues (1994) has demonstrated that a polymorphism in the promoter region of the TNFα gene (termed the TNF2 allele) is associated with higher constitutive and inducible levels of TNFα transcription which alters the clinical course of cerebral malaria. It may be speculated that inter-individual variations in constitutive TNFα production may result in differences in the *in vitro* sensitivity, coupling or expression of the two TNFR subtypes for this cytokine, i.e. individuals who are 'high producers' of TNFα may as a consequence display chronic receptor desensitization. Studies comparing neutrophil responsiveness to the cytocidal effects of TNFα run in parallel with assays of TNFα production by stimulated peripheral

blood mononuclear cells from the same donor may help elucidate whether this is in fact the case. An alternative explanation is that inter-individual variations may exist in terms of receptor affinity or signal transducing capacity for TNFα and its receptors.

When comparing the responsiveness of neutrophils to the pro-apoptotic effect of TNFα, it was observed that cells isolated from the blood of donors where venipuncture was performed >12 weeks after previous donation, the response was very consistent. However, blood obtained from individuals where donations were made more frequently (e.g. 40-80 ml venisected weekly) it was observed that the induction of apoptosis by TNFα was far more variable (data not shown). This led to speculation that following a single large volume blood donation, there is bone marrow release of more juvenile neutrophils which may be more responsive to the cytocidal effects of TNFα; caution must be taken however when interpreting such observations due to the relatively short half-life (about 4 hr) (Price and Dale, 1977) of neutrophils in the circulation. However, with this in mind and the observation that even under optimal conditions only a proportion of the total neutrophil pool undergo apoptosis in response to TNFα led to the design of experiments to address whether neutrophil maturation had a bearing on cellular responsiveness to TNFα. The much more modest and variable increase in peripheral neutrophil count following exertion and the fact that a large proportion of the increase in circulating cells represents release of marginated pulmonary vascular neutrophils rather than marrow release suggested that a steroid neutrophilia protocol was a more sensible strategy.

The treatment of 2 individual donors with Prednisolone (30 mg/day) with white blood cell analysis from whole blood and parallel assessment of TNFα-induced apoptosis allowed us to analyse the effects of long-term glucocorticoid treatment on peripheral blood leukocyte levels and the pro-apoptotic effect of TNFα in neutrophils. In accordance with a previous publication (Schleimer, 1990) glucocorticoid treatment increased the neutrophil counts in both donors, while decreasing eosinophil counts, and the neutrophil killing ability of TNFα was enhanced dramatically following glucocorticoid treatment. While these data are preliminary, the results are consistent and striking and a number of speculations can be made. Contaminating eosinophils, like monocytes, may produce a 'protective factor' which decreases the responsiveness of the neutrophil to the cytotoxic effects of TNFα; since there are less surviving eosinophils following glucocorticoid treatment, the effects of such postulated factors would be diminished. Likewise, the percentage of younger neutrophils will also be greater in the steroid treated subjects, and these younger cells may be more sensitive to the pro-apoptotic effects of TNFα.

Recent data reports that rat bone marrow neutrophils are insensitive to TNFα-mediated apoptosis (Tsuchida et al., 1995), however these cells were harvested from the bone marrow directly, subjected to a harsh extraction protocol, and the resultant yield contained only 60% neutrophils. Finally, it is possible that steroids could increase the ability of TNFα to induce apoptosis in these cells at a transcription/translation level by increasing receptor affinity and signal transducing

ability and thereby causing up regulation of the TNFα killing effect or down regulation of a cell survival effect.

In light of previous demonstrations that the pro-apoptotic effect of TNFα in other cell systems is dependent on a specific set of culture conditions (Beyaert and Fiers, 1994), a series of experiments were designed to determine whether factors characteristic of our system influence the responsiveness of the neutrophil to TNFα, with the particular objective of determining whether the efficacy of the pro-apoptotic effect of this cytokine could be enhanced. Examination of the influence of cell concentration on the action of TNFα at 6 hr revealed that the pro-apoptotic effect was most marked at 2.5-5x10^6 cells/ml; this may reflect production of a neutrophil (or even eosinophil/monocyte)-derived protective factor(s) which at higher cell densities is produced in saturating concentrations, or reflect the loss of any potential surface effects as the cells no longer settle in a monolayer on the flexiwell surface.

In view of the fact that TNFR55 and TNFR75 can be proteolytically cleaved from the cell surface and thus form soluble TNFα binding proteins (TNFR55-BP and TNFR75-BP; Olsson et al., 1989, Seckinger et al., 1989, Engelmann et al., 1989) that have been detected in human serum (Petree et al., 1988), the effect of serum withdrawal on the pro-apoptotic effect of TNFα was assessed. In our experiments however, neither serum withdrawal or substitution with 10% fetal calf serum had any influence on the pro-apoptotic effect of TNFα, implying that neither the intra-donor variation in the pro-apoptotic neutrophil responsiveness to TNFα, or the observation

that only a proportion of cells undergo apoptosis in response to this cytokine, can be attributed to inhibition of TNFα action by TNF-BPs in the serum. Moreover, these data eliminate the possibility that other serum factors, such as the proteinase α_2-macroglobulin, which has been shown to bind a number of cytokines (LaMarre et al., 1991), are binding to or having a major neutralizing effect on the activity of exogenously added TNFα. This would be more appropriately studied however using an EC_{50} concentration of TNFα which would provide a far more sensitive index of the capacity of serum factors to influence TNFα-stimulated apoptosis.

The loss of the pro-apoptotic effect of TNFα when cells were cultured for 6 hr prior to cytokine addition was considered to be most likely attributable to cell adhesion since in shorter (30 min) pre-incubation experiments the cytotoxic effect of TNFα was partially preserved, especially when the cells were pre-incubated in suspension by gentle agitation in polypropylene tubes; the influence of cell adhesion could be examined in more detail by assessing the ability of TNFα to induce apoptosis in pre-incubated non-adherent cells e.g. in the presence of CD18 $F(ab')_2$ monoclonal antibodies. The loss of the pro-apoptotic effect of TNFα under adherent conditions may well reflect the major decline in TNFR55 and TNFR75 expression observed over this period as demonstrated in Chapter 5 (5.2.3B). This hypothesis is supported by data from Tsuchida and colleagues (1995) who showed a correlation between TNF affinity (although not number) and TNFα-induced apoptosis in rat neutrophils harvested from bone marrow, peripheral blood and the inflamed peritoneum. This hypothesis does not however account for the enhanced inhibitory effect of TNFα

observed at 20 hr under these conditions. In view of data from a number of groups, including our own, demonstrating that adhesion primes the neutrophil for enhanced respiratory burst activity and converts TNFα into a full secretagogue for superoxide anion generation (Nathan, 1987, Richter et al., 1989, Nathan et al., 1989, Dri et al., 1991, Nathan and Sanchez, 1990; ER Chilvers and AM Condliffe, personal communication), it is reasonable to speculate that the enhanced late apoptotic inhibitory effect of TNFα may reflect direct triggering of the release of an anti-apoptotic factor(s) e.g. LTB_4 (Ishii et al., 1992).

Of relevance to the inflamed site, the ability of TNFα to accelerate apoptosis was completely lost if neutrophils were primed with PAF (1 μM, 10 min) prior to cytokine addition. Moreover, as demonstrated in Chapter 5 (5.2.3B) this effect was not secondary to decreased TNFR55 or TNFR75 expression. Although a previous study has reported that PAF induces a 50% decline in total TNFR expression using ^{125}I-TNFα binding, this effect was obtained only following a 2 hr rather than 5 min incubation period and employed PAF at a supra-pharmacological concentration of 100 μM (Schleiffenbaum and Fehr, 1990). It is possible however that PAF influences receptor coupling rather than expression and/or that TNFα is sensitive to PAF-induced protease release. Studies employing the long-acting PAF receptor antagonist UK-74,505 (Alabaster et al., 1991), or other rapidly-acting priming agents such as IL-8 (10 min; Daniels et al., 1992) may help elucidate whether this PAF-mediated attenuation is a general consequence of the activation status of the neutrophil (i.e. priming) or a specific receptor-mediated event.

A further study (Hachiya et al., 1995) has demonstrated a dose-dependent albeit partial inhibition of TNFα-stimulated apoptosis in human neutrophils by LPS. These experiments employed very high concentrations of LPS (1-100 μg/ml) used over a short pre-incubation time (30 min; maximal priming with LPS requires 120 min; Guthrie et al., 1984) and hence it is most likely that the attenuated pro-apoptotic effect of TNFα observed with LPS was secondary to a modest reduction in expression of both receptor subtypes as previously reported (Porteu and Nathan, 1990).

Of interest, a recent study by Tsuchida et al., (1995) compared the sensitivity of rat neutrophils, obtained from various tissues and sites, to the pro-apoptotic effect of TNFα *in vitro*. The study showed that while peripheral blood neutrophils were responsive to the pro-apoptotic effect of TNFα, peritoneally exudated neutrophils (PEN) and inflammatory peripheral blood neutrophils, both obtained after an intra-peritoneal (i.p.) injection of proteose peptone (a neutrophil chemoattractant) were resistant to the cytotoxic effect of this cytokine. Although these differences could not be ascribed to variations in TNFα receptor number, the neutrophils from these different sources did show differences in the affinity of TNFα binding with a higher K_d observed in the non-responsive cells. Kinetic analysis of the inflammatory peripheral blood neutrophils revealed that the sensitivity to TNFα started to decrease at 3 hr after i.p. injection, was lowest at 12 hr and was almost fully restored after 52 hr. Of note, an inverse correlation was observed between cell number and cell sensitivity to TNFα. One other fascinating finding of this study was that peritoneal

neutrophils elicited by i.p. injection of physiological saline retained their sensitivity to TNFα.

In another recent study it was demonstrated that exudated human salivary neutrophils were unresponsive to TNFα (and G-CSF), as assessed by priming for fMLP-stimulated superoxide anion generation, as compared to peripheral blood neutrophils examined in parallel. This effect was again associated with a decrease in TNF receptor affinity but not in receptor density (Niwa et al., 1996). These studies and other reports, demonstrating that treatment of neutrophils with priming and/or activating agents such as fMLP or C5a decrease TNFR density and affinity (Solomskin et al., 1994), highlight the complex and delicate interplay that must occur between cytokines in controlling neutrophil behaviour within an inflammatory focus *in vivo*.

Finally, to explore whether the efficacy of the pro-apoptotic effect of TNFα *in vitro* could be enhanced by anti-inflammatory cytokines, we examined the effect of IL-10 on TNFα-stimulated neutrophil apoptosis. IL-10 exerts potent anti-inflammatory effects *in vivo*, which have been attributed to inhibition of cytokine production by monocytes/macrophages (De Waal Malefyt et al., 1991, Gerard et al., 1993, Howard et al., 1993); in this context, TNFα-dependent mechanisms are diminished (Lo et al., 1992). In addition, neutrophil production of regulatory mediators such as cytokines (e.g. TNFα and GM-CSF), and the IL-1 receptor antagonist, are also subject to regulation by IL-10 (Cassatella et al., 1993).

In our studies IL-10 had no influence on the rate of constitutive or TNFα-stimulated neutrophil apoptosis. Of interest, in a recent *in vivo* study in rats by Cox (1996), it was demonstrated that while intra-tracheal administration of IL-10 did not modulate the onset or peak of neutrophil accumulation in response to intra-tracheal LPS challenge, this cytokine did promote the clearance of recruited neutrophils. During *ex-vivo* culture of cells obtained by bronchoalveolar lavage (BAL), neutrophil apoptosis and subsequent macrophage ingestion was accelerated in the BAL of IL-10-treated rats. Exogenously added IL-10, however, did not influence the rate of apoptosis of neutrophils prepared from rat peripheral blood neutrophils but did inhibit LPS-mediated inhibition of cell death in a concentration-dependent manner.

These data indicate the potential for IL-10 to act in concert with other inflammatory cytokines *in vivo* to up-regulate the rate of neutrophil apoptosis. The inability of IL-10 to influence TNFα-mediated apoptosis however suggests that the activity of this cytokine is not directly pro-apoptotic, but rather functions to down-regulate the inhibition of apoptosis by other inflammatory cytokines, either by inhibiting their production, or by modulating their ability to enhance survival.

Chapter 5: Role of the TNFR55 and TNFR75 subtypes in the regulation of neutrophil apoptosis by TNFα

5.1 Introduction

Two distinct receptor subtypes for TNFα with molecular masses of 55 kD (TNFR55) and 75 kD (TNFR75) have now been identified (Hohmann et al., 1990, Brockhaus et al., 1990), and human and mouse cDNAs corresponding to both receptor types have been isolated and characterized (Loetscher et al., 1990, Schall et al., 1990, Smith et al., 1990, Lewis et al., 1991, Goodwin et al., 1991). These receptors are members of a superfamily of receptors referred to as the NGF/TNF receptor family which are characterized by functional trimerization and the presence of one to six cysteine-rich repeats of approximately 40 amino acids in the extracellular domain that provide the motif for binding to shared structures in the ligand (section 1.3.7). TNFR55 is found on almost all cell types whereas TNFR75 has a far more restricted distribution with expression on cells of haematopoietic lineage and endothelium (Hohmann et al., 1989, Brockhaus et al., 1990, Shalaby et al., 1990, Porteau et al., 1991, Mackay et al., 1993).

The extracellular domains of the two human TNFRs are 28% identical and have no more homology to each other than to other members of the NGF/TNF receptor superfamily. Likewise, there is no significant homology between the intracellular domains of the two TNFRs, indicating that these receptors almost certainly employ

different signalling mechanisms (Lewis et al., 1991). TNFR55 contains a classical death-domain sequence (Tartaglia et al., 1993a) and has been reported to be the sole mediator of the TNFα-mediated death signal in most non-haematopoietic cell types. Despite this, the TNFR75, which lacks such a death domain sequence, has been proposed to play a key role in mediating TNFα-induced cytotoxicity in murine $CD8^+$ peripheral T-cells (Zheng et al., 1995). Hence the aim of the work presented in this chapter was to determine the TNFR-dependency of the TNFα pro-apoptotic effect in human neutrophils.

5.2 Results

5.2.1 Effect of TNFR55 agonist polyclonal antibodies on early neutrophil apoptosis

The potential role of TNFR55 in mediating the early pro-apoptotic effect of TNFα in neutrophils was initially investigated using a total goat IgG anti-human soluble TNFR55 activating polyclonal antibody. This antibody has been reported to interact with cell surface TNFR55 and, in the presence of actinomycin D, induce cytotoxicity in human A549 cells (EC_{50} 5-10 μg/ml) and murine L929 cells (EC_{50} 10-15 μg/ml; R & D systems catalogue, 1996). Figure 5.2.1 however demonstrates the inability of this antibody to mimic the pro-apoptotic effect of 12.5 ng/ml TNFα following a 6 hr incubation. The functional activity of this antibody was confirmed in experiments demonstrating its ability (at a concentration of 1 μg/ml) to mimic TNFα-induced priming of fMLP (100 nM)-stimulated superoxide anion generation in suspension neutrophils (E Kitchen, Ph D Thesis, 1997). These data imply that activation of the TNFR55 subtype alone may be insufficient for transmission of a TNFα-mediated death signal in neutrophils.

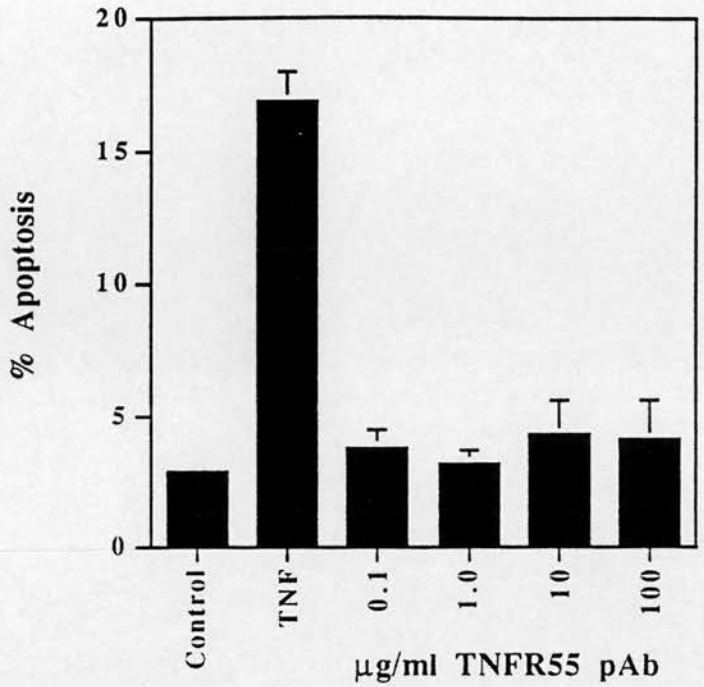


Figure 5.2.1 Effect of TNFR55 agonist polyclonal antibodies on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils ($5x10^6$/ml) were cultured in serum-supplemented Iscove's MDM alone (control) or in identical medium containing: (final concentration) TNFα (12.5 ng/ml) or total goat IgG anti-human TNFR55 polyclonal antibody (pAb, 0.1-100 μg/ml). Neutrophils were harvested following 6 hr in culture and apoptosis assessed morphologically. Data represent mean ± SEM of triplicate determinations in a single representative experiment of 3.

5.2.2 Effect of TNFR55 blocking monoclonal antibodies on the pro-apoptotic effect of TNFα in neutrophils

Further investigation into the involvement of TNFR55 in the pro-apoptotic effect of TNFα in neutrophils was performed using a mouse anti-human IgG_1 TNFR55 blocking monoclonal antibody. Pre-incubation of human neutrophils for 30 min with this antibody completely abrogated the pro-apoptotic effect of TNFα at 6 hr without affecting the basal rate of apoptosis (figure 5.2.2), indicating a requirement for TNFR55 activity in TNFα-signalling for neutrophil death. The relatively small pro-apoptotic effect of TNFα observed in these experiments (approximately 15% at 6 hr) compared to data presented in Chapter 3 (e.g. figure 3.2.2) reflects the need to pre-incubate cells for 30 min prior to exposure to TNFα (see Chapter 4). The specificity of the antibody for the TNFR55 was confirmed in additional experiments where TNFα-induced priming of fMLP (100 nM)-stimulated superoxide generation was completely blocked (E Kitchen, Ph D Thesis, 1997).

5.2.3A Flow cytometric analysis of TNFR55 and TNFR75 on human neutrophils

Flow cytometric analysis of TNFR55 and TNFR75 in freshly isolated human neutrophils demonstrated a single population of TNFR55 and TNFR75 positive cells (figure 5.2.3A) with mean fluorescence values of 4.06 ± 0.26 and 5.40 ± 0.53, respectively (CD2 control values 1.50 ± 0.10, n=9). Moreover, TNFR55 and TNFR75 were expressed in approximately equal densities (figure 5.3.3B).

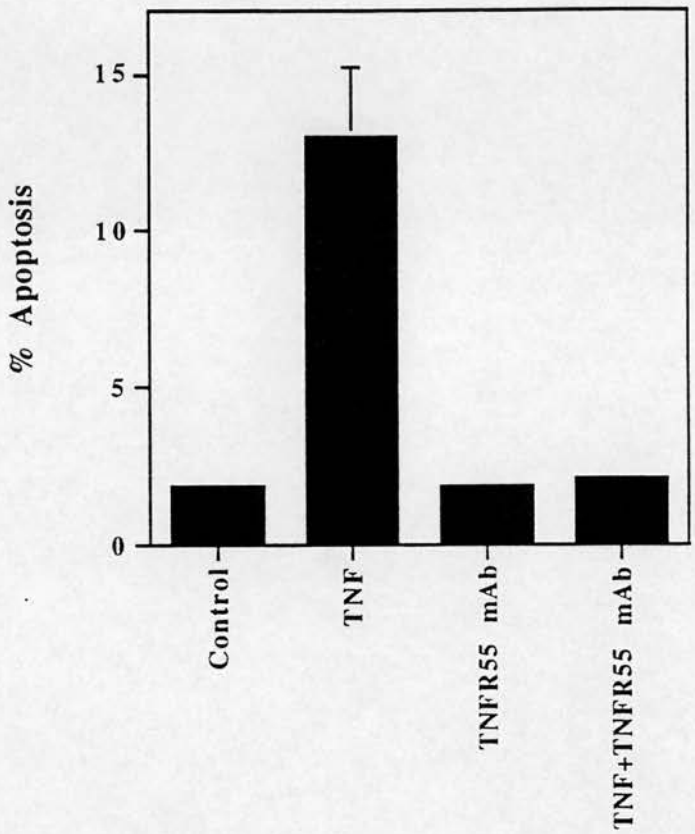


Figure 5.2.2 Effect of TNFR55 blocking monoclonal antibodies on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils ($5x10^6$/ml) were pre-incubated with 28 μg/ml mouse IgG_1 anti-human TNFR55 monoclonal antibody (mAb) in serum-supplemented Iscove's MDM for 30 min at 37°C prior to culture the presence or absence of 12.5 ng/ml TNFα. Neutrophils were harvested following 6 hr in culture and apoptosis assessed morphologically. Data in mean ± SEM of 6 determinations from 2 independent experiments of 4; where not shown, SEM values are <2% of means.

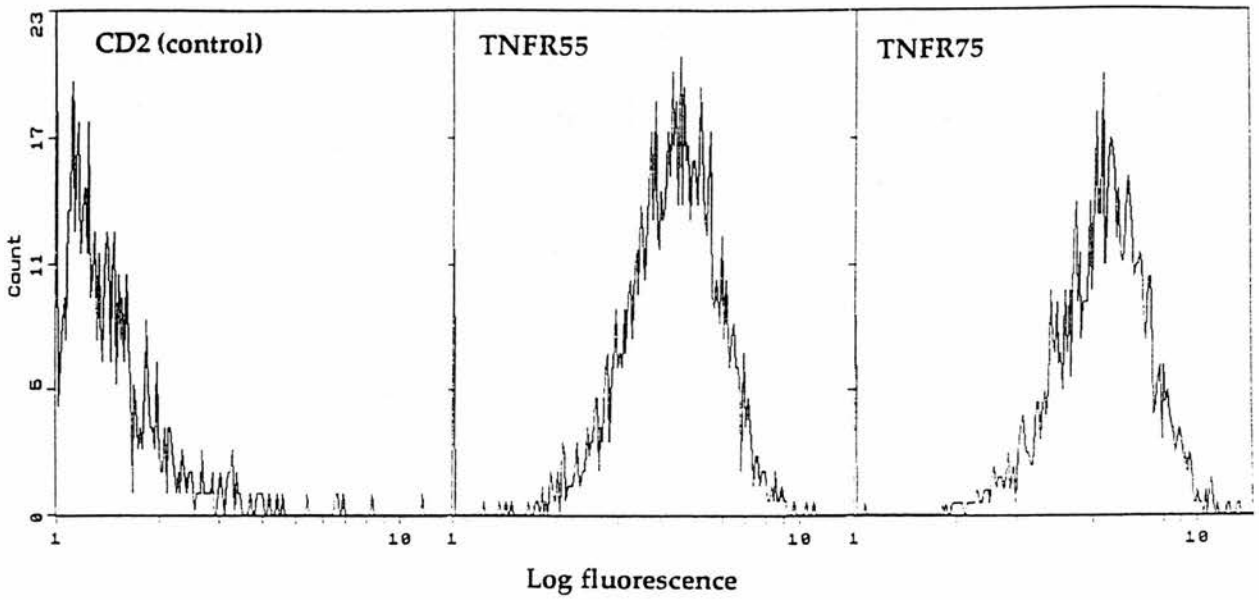


Figure 5.2.3A Flow cytometric analysis of TNFR55 and TNFR75 in human neutrophils

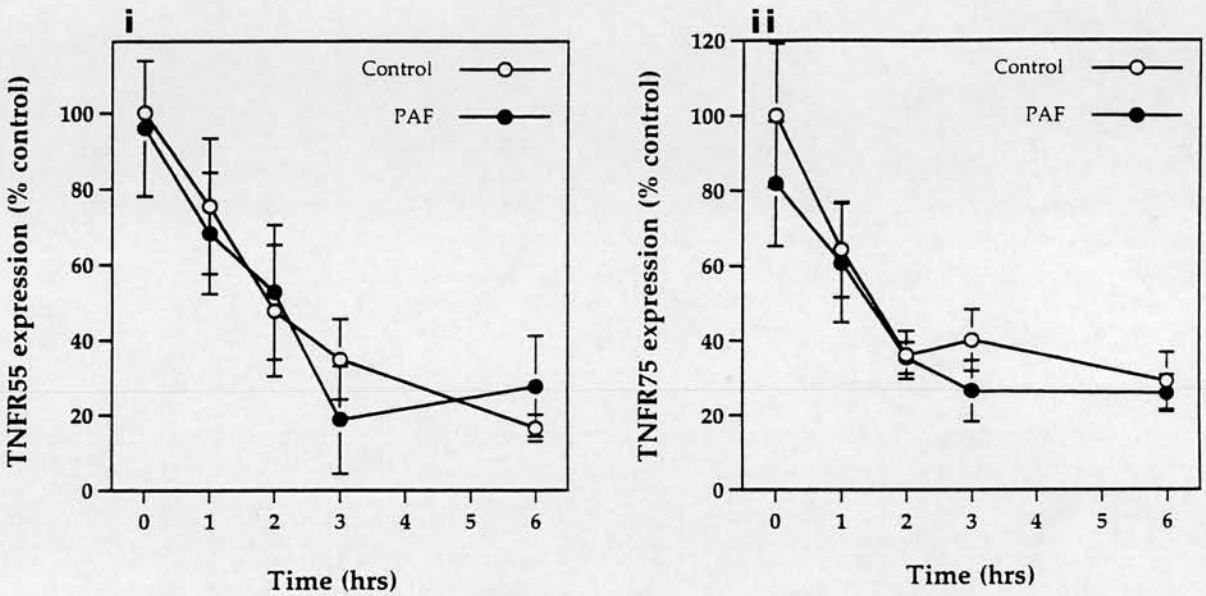
Freshly purified human neutrophils were incubated at 4°C for 30 min with a saturating concentration of mouse anti-human TNFR55, TNFR75, or CD2 (negative control) mAbs and subsequently with FITC-conjugated goat anti-mouse immunoglobulin (30 min). Histograms represent profiles of cell count against log fluorescence and are representative examples from 9 separate experiments

5.2.3B Time course for TNFR55 and TNFR75 expression in human neutrophils in the presence and absence of PAF

Flow cytometric analysis of TNFR55 and TNFR75 expression in neutrophils incubated for 0,1,2,3 or 6 hr demonstrated a time-dependent decrease in both TNFR55 and TNFR75 expression (figure 5.2.3B); however, pre-incubation of cells with PAF (1 μM) for 5 min, which completely abolished the ability of TNFα to induce apoptosis at 6 hr (section 4.2.7), had no effect on either TNFR55 or TNFR75 expression (figure 5.2.3B).

5.2.4 Effect of TNFR75 blocking monoclonal antibodies on the pro-apoptotic effect of TNFα on neutrophils

In view of the fact that neutrophils cells express TNFR55 and TNFR75 in approximately equal numbers, and that agonistic TNFR55 antibodies failed to induce apoptosis in these cells, the potential role of the TNFR75 in mediating the pro-apoptotic effect of TNFα in neutrophils was investigated using a rat IgG_{2b} anti-human TNFR75 monoclonal antibody. As shown in figure 4.2.6, pre-incubation of neutrophils for 30 min with this antibody also caused a near complete (81.2 ± 7.0%) abrogation of the pro-apoptotic effect of TNFα in neutrophils without affecting the basal rate of apoptosis. Failure of an isotype matched rat anti-human IL-2 receptor antibody to affect this response (figure 4.2.6) confirmed that the inhibition observed with the former antibody was specific to interaction with TNFR75. Additional



5.2.3B Time course for TNFR55 and TNFR75 expression in human neutrophils in the presence and absence of PAF

Human neutrophils ($5x10^6$/ml) were pre-incubated in serum-supplemented Iscove's MDM in the presence or absence of PAF (1 μM) for 5 min at prior to incubation for 0, 1, 2, 3 or 6 hr in flexiwell plates at 37°C. At these time points TNFR55 (figure 5.2.3B(i) and TNFR75 (figure 5.2.3B (ii) expression was quantified by flow cytometry as detailed in Materials and Methods (2.2.6). Data represent % of specific receptor expression values obtained in freshly isolated cells with each point being the mean ± SEM of 3 separate experiments each performed in triplicate.

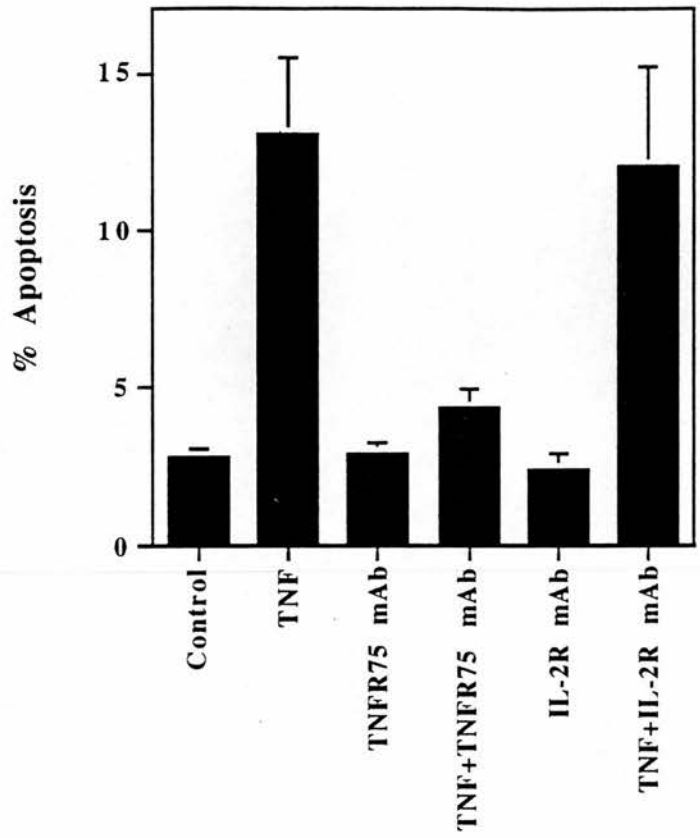


Figure 5.2.4 Effect of TNFR75 blocking monoclonal antibodies on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils ($5x10^6$/ml) were pre-incubated in serum-supplemented Iscove's MDM with 28 μg/ml rat IgG_{2b} anti-human TNFR75 monoclonal antibody (mAb) or isotype matched rat anti-human IL-2 receptor antibody (IL-2R mAb, negative control) for 30 min at 37°C prior to culture in the presence or absence of 12.5 ng/ml TNFα as indicated. Neutrophils were harvested following 6 hr in culture and apoptosis assessed morphologically. Data represent mean ± SEM of 4 experiments, each performed in triplicate.

experiments demonstrated that the anti-TNFR75 antibody had no effect on TNFα-mediated priming of fMLP (100 nM)-stimulated superoxide generation in neutrophils (E Kitchen, Ph D Thesis, 1997). These data imply that co-activation of TNFR75 is required for TNFα-signalling for apoptosis in neutrophils.

5.2.5 Effect of TNFR55-selective mutants on early neutrophil apoptosis

In the absence of available TNFR75 agonistic antibodies, complementary experiments were performed in collaboration with Dr. Jeffrey A. J. Barbara (Renal Unit, Flinders Medical Centre, Bedford Park, South Australia 5042) to assess the effects of the TNFR55-selective TNFα agonistic mutants E146K and R32W-86T and the TNFR75-selective mutant D143F (Barbara et al., 1994) on neutrophil apoptosis. The former TNFR55-selective peptides have a 3300- and 5000-fold lower affinity for the TNFR75 receptor with only marginal (2- and 2.2-fold) reduction in TNFR55 affinity whereas the TNFR75 ligand D143F displays a 30-fold reduced affinity for TNFR75 and no binding to TNFR55 (Van Ostade et al., 1994, Loetscher et al., 1993).

Figure 4.2.5 demonstrates the ability of both TNFR55 mutants to induce apoptosis (as assessed by morphological criteria), albeit with a lower potency than recombinant TNFα (EC_{50} values, ng/ml: wild type TNFα, 5.0; E146K, 82.7; R32W-S86T, 69.5; both $p < 0.01$ compared to TNFα). The TNFR75 selective agonist (D143F) did not induce apoptosis even at a concentration of 100 ng/ml. These data imply that one

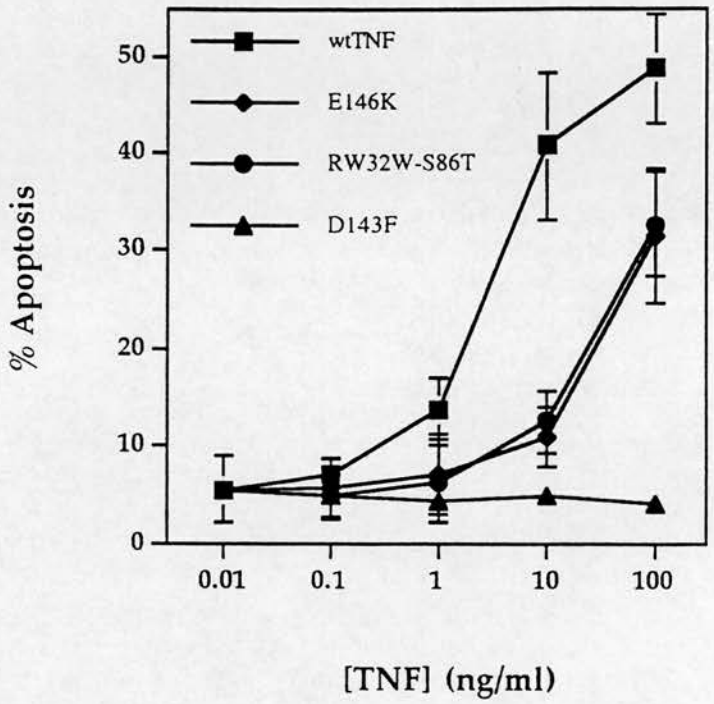


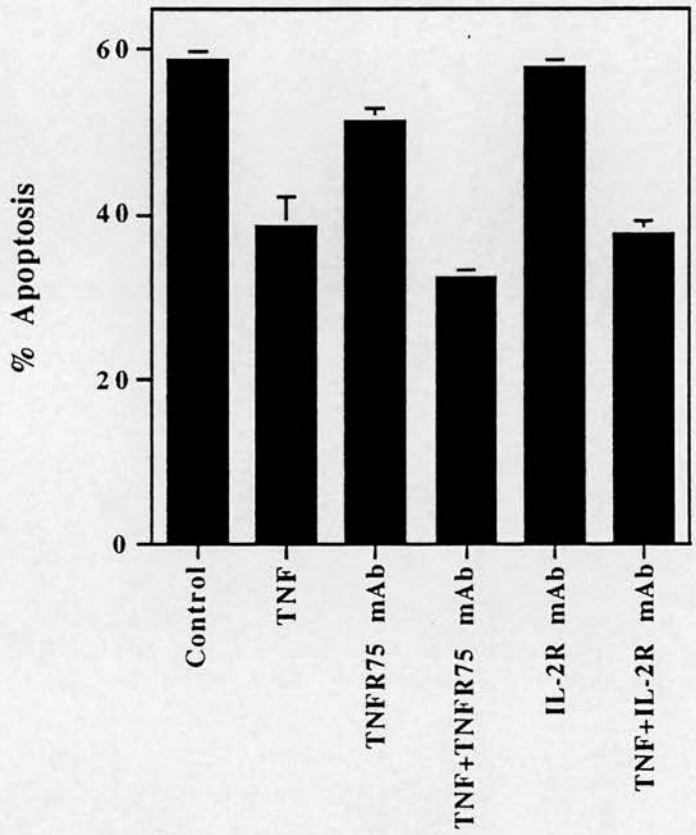
Figure 5.2.5 Effect of TNFα receptor selective muteins on human neutrophil apoptosis

Human neutrophils were incubated in RPMI-1640 medium supplemented with 0.1% bovine serum albumin (BSA) with 0.01-100 ng/ml of wild-type TNFα (wtTNFα, squares), the TNFR55-selective muteins R32W-S86T (circles) or E146K (diamonds), and the TNFR75-selective mutein D143F (triangles). Apoptosis was assessed morphologically after 3 hr. Data represent mean ± SEM of three experiments, each performed in triplicate (experimental data provided by Dr. Jeffrey Barbara).

role of the TNFR75 is to facilitate the ability of TNFR55 to induce apoptosis in human neutrophils.

5.2.6 Effect of TNFR75 blocking antibody on TNFα-mediated inhibition of apoptosis at 20 hr

The involvement of TNFR75 in mediating the later, inhibitory effect of TNFα in neutrophils was also evaluated using the rat anti-human IgG_{2b} monoclonal antibody which blocked the pro-apoptotic effect of TNFα as described in 4.2.4. Figure 4.2.6 demonstrates that pre-incubation of neutrophils for 30 min with this antibody, or the isotype matched IL-2R control antibody, had no influence on the late inhibitory effect of TNFα on apoptosis observed at 20 hr. This lack of effect of the TNFR75 blocking antibody on the late inhibition of neutrophil apoptosis by TNFα was not a result of loss of functional activity since flow cytometric analysis of antibody binding was identical for fresh antibody and for antibody incubated for 20 hr in serum-supplemented medium (data not shown). These data indicate that, in contrast to its essential role in mediating the early pro-apoptotic effect of TNFα, TNFR75 does not appear to be involved in signaling the later anti-apoptotic effect of TNFα. In equivalent experiments designed to assess the role of TNFR55 in this system the TNFR55 blocking antibody itself appeared to inhibit slightly the basal rate of apoptosis at 20 hr excluding further analysis (data not shown).



5.2.6 Effect of TNFR75 blocking monoclonal antibody on TNFα-mediated inhibition of neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were pre-incubated with 28 μg/ml rat IgG_{2b} anti-human TNFR75 monoclonal Ab (mAb) or isotype matched rat anti-human IL-2 receptor mAb (IL-2R mAb, negative control) in serum-supplemented Iscove's MDM for 30 min at 37°C prior to culture in the presence or absence of 12.5 ng/ml TNFα as indicated. Neutrophils were harvested following 20 hr in culture and apoptosis assessed morphologically. Data represent mean ± SEM of triplicate determinations in a single representative experiment of 2.

5.3 Discussion

The absolute number of TNFRs on the cell surface of human neutrophils is rather low, falling within the range of 500-6000 (Shalaby et al., 1987, Larrick et al., 1987, Schleiffenbaum and Fehr, 1990, Porteu and Nathan, 1990). In the work presented in this chapter we have demonstrated that TNFR55 and TNFR75 are expressed in approximately equal densities on freshly isolated human peripheral blood neutrophils. Unlike this finding in agreement with the previous report of Barbara and co-workers (1994), a study by Abe et al., (1995) using in-house agonistic rabbit anti-human TNFR polyclonal antibodies suggested that the TNFR75 was the predominant receptor expressed on these cells. An earlier study by Lantz et al., (1994) demonstrated shedding of TNFRs in human neutrophils following adherence or activation by both PMA and fMLP, which was more pronounced for TNFR55. The neutrophil isolation technique used in our studies results in minimal priming which can occur as a consequence of other cell preparation methods (e.g. Ficoll gradient separation or red cell lysis, Haslett et al., 1985). Since shape-change and aggregation accompany neutrophil priming, one might speculate that disparities in the literature with regard to the relative expression of the two TNFRs may be a consequence of selective receptor shedding occurring during different cell preparation methods. The time-dependent decrease in TNFR55 and TNFR75 during neutrophil culture is consistent with the demonstration of adherence-induced TNFR shedding by Lantz et al., (1994), and is discussed in detail in Chapter 4.

TNFα activates TNFR55 and TNFR75 by oligomerization through the trimeric ligand. The ability of TNFR-specific agonistic antibodies to mimic TNFα activities is evidence that the sole function of TNFα is clustering of its receptors and that no additional activities of this cytokine are required after internalization of the ligand-receptor complex (Espevik et al., 1990). The k_d values of TNFR55 and TNFR75 for TNFα are approximately 0.5 nM and 0.1 nM respectively (Loetscher et al., 1990, Schall et al., 1990, Smith et al., 1990).

A number of studies have attempted to examine the function of TNFR55 and TNFR75 in neutrophils. These studies have employed a variety of selective mono- and polyclonal blocking and agonist antibodies together more recently with specific agonist mutant proteins. Very few of these studies however have used more than one type of reagent and there have been no comparative studies examining the effects of these agents. Hence, although most authors have demonstrated a major role for TNFR55 in priming of respiratory burst activity and degranulation, considerable debate remains about the functional role of TNFR75. Hence Abe and colleagues (1995) using in-house polyclonal antibodies claimed that TNFR55 is the only receptor subtype involved in TNFα-induced priming of elastase, lactoferrin and superoxide release. In contrast, Barbara et al., (1994) using selective mutein agonists, concluded that TNFR75 activation facilitates TNFR55 function. Similarly, in adherent neutrophils, where TNFα functions as a full secretagogue (Nathan et al., 1987, Richter et al., 1989, Nathan et al., 1989, Dri et al., 1991), Meganazzi and co-workers (1994) describe TNFR55 as being fully competent to induce superoxide

generation whereas Richter et al., (1995) report an absolute requirement for TNFR75 activation for this response. Preliminary data from our group employing selective TNFR55 and TNFR75 blocking monoclonal antibodies indicate that, at least in suspension cells, TNFR75 is not involved in TNFα-induced neutrophil priming (E Kitchen, Ph D Thesis, 1996).

In these studies we have demonstrated using receptor blocking antibodies that dual activation of TNFR55 and TNFR75 is required for TNFα-mediated apoptosis in human neutrophils. Moreover, the effectiveness of the TNFR55-selective mutants E146K and R32W-S86T to induce apoptosis in these cells implies that TNFR75 most likely functions to facilitate a death signal primarily initiated via TNFR55. These data regarding the pro-apoptotic effects of TNFα are similar to results obtained in studies using the TNFR-specific muteins to investigate the relative receptor-dependency of the pro-inflammatory activities of TNFα (Barbara et al., 1994). In this report the author suggested that the inability of the TNFR75 receptor mutein D143F on its own to stimulate neutrophil functions may be due to its 30-fold reduction in affinity for TNFR75 compared to wild-type TNFα which may result in suboptimal receptor clustering (a pre-requisite for TNFα-signalling) especially at low receptor numbers. In support of this, D143F induced GM-CSF production in PC60-hTNFR75^{+} cells which have a higher expression of this receptor.

Involvement of TNFR75 in TNFα-mediated death appears to be restricted to haematopoietic cells and was initially thought to reflect the ability of the higher

affinity TNFR75 to present TNFα to the lower affinity TNFR55 via a 'ligand passing' effect (Tartaglia et al., 1993b). However, our results demonstrating (i) the inability of TNFR55 agonistic antibodies to induce neutrophil apoptosis while maintaining full agonist activity in priming of fMLP-stimulated superoxide generation, and (ii) data presented in Chapter 6 (section 6.2.1), excluding involvement of the sphingomyelinase-ceramide pathway in the induction of neutrophil cell death, a principal signalling route associated with TNFR55, suggests that, at least in the neutrophil, this is not the case. Moreover, data in PC60 T-cell hybridomas, where co-expression of high levels of TNFR75 in addition to low levels TNFR55 is likewise required to observe TNFα-stimulated apoptosis, have excluded such a mechanism (Vandenabeele et al., 1995).

The involvement of TNFR75-derived intracellular signals in the death effect of TNFα is supported by the recent demonstration that TNFα can induce T-cell receptor-induced apoptosis in *p55*$^{-/-}$ mice indicating that under certain circumstances TNFR75 can function alone to induce apoptotic cell death (Zheng et al., 1995) and similar conclusions have been reached in HeLa cells using TNFR75-selective antibodies (Bigda et al., 1994). The precise mechanism whereby TNFR75 initiates apoptosis or permits TNFR55-induced death remains uncertain however, not least because this receptor has a relatively short cytoplasmic domain with no intrinsic kinase activity or death domain sequence (as found in TNFR55 and Fas (Tartaglia et al., 1993a). However, the recent discovery of a TNFR75-associated kinase which

phosphorylates both TNFRs provides a potential mechanism whereby TNFR75 could facilitate TNFR55 intracellularly (Dornay et al., 1994).

Weiss and co-workers (1997) have recently indicated a role for the TNF receptor-associated factor-2 (TRAF2) in the cross-talk between TNFR55 and TNFR75 that underlies TNFα-mediated cytotoxicity in HeLa transfectants expressing wild-type TNFR75. In these studies, selective stimulation of TNFR75 with agonist mAbs was insufficient to induce apoptosis, however TNFR55-mediated cytotoxicity was enhanced 1000-fold by co-stimulation of TNFR75. Moreover, the use of selective agonistic Abs to induce dual receptor stimulation ruled out that the enhancement observed was a result of ligand passing. A role for TRAF2 in this receptor synergy model was suggested by the inability of HeLa TNFR75 deletion mutants, which lacked the TRAF2 binding, to potentiate TNFR55-mediated cytotoxicity in these cells. Although TRAF2 appears to mediate both TNFR55 and TNFR75-induced activation of NFκB, as well as that related to CD40 receptor stimulation (Rothe et al., 1995b, Hsu et al., 1996a), several lines of evidence suggest that the TNFR75-mediated potentiation of TNFR55-stimulated cytotoxicity observed was independent of activation of this transcription factor; (i) while co-stimulation of both receptors resulted in a 1000-fold increase in cytotoxicity over that observed with TNFR55 stimulation alone, the induction of NFκB-dependent responses (IL-6 and manganese superoxide dismutase generation) was additive, (ii) the TNFR75-mediated co-operativity was only effective in the presence of protein synthesis inhibitors, making association with NFκB-dependent gene expression unlikely and (iii), there are now

several reports in the literature to support a protective role for NFκB in TNFα-mediated apoptosis (Beg and Baltimore, 1996, Wang et al., 1996, Van Antwerp et al., 1996, Liu et al., 1996).

An independent pro-apoptotic role for TRAF2 is supported by studies demonstrating that CD30 and CD40, which can induce cytotoxicity under certain conditions, can also bind TRAF2 (Mosialos et al., 1995, Cheng et al., 1995, Lee et al., 1996). In the case of CD30-mediated TCR-dependent cell death of T cell hybridomas, the receptor binding site of TRAF2 is also necessary for the induction of cytotoxicity (Lee et al., 1996). Moreover, synergistic actions of TNFR55 and CD40 have been demonstrated in HeLa cells (Hess and Engelmann, 1996).

As the direct functional role for TRAF2 in TNFR75-mediated potentiation of TNFR55-dependent cytotoxic signalling has yet to be defined, it cannot be overlooked that TRAF2 may not be involved in this process and that the TRAF2 binding site for TNFR75 may bind or co-operate in the binding of as yet another unidentified factor(s) responsible for apoptotic signalling. Finally, of interest, the TNFR55-associated protein TRADD, which is necessary for TNFR55-mediated apoptosis (Hsu et al., 1995, 1996b), interacts directly with TRAF2 in intact cells and therefore affords a further direct link between TNFR55 and TNFR75 signalling (Hsu et al., 1996a).

Chapter 6: Signalling pathways in TNFα-stimulated apoptosis in human neutrophils

6.1 Introduction

In the previous chapters we have established and characterized the ability of TNFα to induce apoptosis in human neutrophils at early time points. Moreover, we have demonstrated that this pro-apoptotic effect of TNFα is mediated via an unconventional route whereby the TNFR75 facilitates and permits cell death mediated by TNFR55, which contains the classical death-domain sequence (Tartaglia et al., 1993a). There are now a number of reports in the literature which describe studies designed to investigate the role of signalling pathways in TNFα-mediated cytotoxicity in various cell types, and these are summarized in table 6.1.1.

While it is currently difficult to provide a unifying hypothesis from such data, the general theme emerges that inhibition of protein kinase (PK) activity, be it PKC, protein tyrosine (PTyr)K or cyclic nucleotide-dependent kinases (PKA, PKG), and/or concurrent activation of ceramide generation, may underlie TNFα-stimulated apoptosis. In the neutrophil such a hypothesis would be supported by data indicating the majority of agents that activate cells and switch on one or more of these kinase pathways appear to delay apoptosis and also existing results demonstrating that pharmacological activation of the PKC, PKA and PKG pathways also enhance

CELL TYPE	PROPOSED SIGNALLING ROUTE FOR TNFα-INDUCED CYTOTOXICITY
U937	TNFα-induced apoptosis was mimicked by C_2-ceramide and the pro-apoptotic effects of both agents were prevented by the pharmacological PKC activator, PMA (Obeid et al., 1993).
U937	Calyculin A and okadaic acid, protein phosphatase inhibitors, augmented TNFα-induced apoptosis in U937 cells and reversed TNFα resistance in U9-TR variants (Wright et al., 1993).
U937, HL-60, L929, WEHI-164	TNFα-induced apoptosis was mimicked by C_8-ceramide and neutral sphingomyelinase (Jarvis et al., 1994).
Human neutrophils	TNFα-induced apoptosis *in vitro* was mimicked by sphingosine, which is a potent inhibitor of PKC (Ohta et al., 1994).
Murine A9 fibroblasts	TNFα-induced cytotoxicity in the presence of cycloheximide was inhibited (by approximately 50%) by the protein tyrosine kinase inhibitor AG1288 (Novogrodsky et al., 1994).
Mouse hepatocytes	TNFα-induced apoptotic and secondary necrotic liver injury in GalN-sensitized mice *in vivo* was completely prevented by pharmacological doses of NO donor sodium nitroprusside (Bohlinger et al., 1995).
Rat hepatocytes	TNFα-induced apoptosis in the presence of actinomycin-D *in vitro* was inhibited by NO donor SNAP (Kim et al., 1997).

Table 6.1.1 Proposed signalling routes for TNFα-stimulated apoptosis in various cell models

(PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; GalN, D-galactosamine; NO, nitric oxide; SNAP, S-nitroso-N-acetylpenicillamine).

neutrophil survival (MKB Whyte, Ph D Thesis, 1993, Rossi et al., 1995, Cousin et al., 1997; TW Wong, AG Rossi and ER Chilvers, personal communication).

To begin to examine the potential signalling pathway(s) involved in mediating the TNFα death signal in neutrophils, selective pathway inhibitors or cell-permeable mimetics that block TNFα-mediated cytotoxicity in other cell models were exploited.

In view of previous reports demonstrating that in certain cell lines, in particular the murine fibrosarcoma L929 cell line, the cytotoxic effects of TNFα could be abrogated by culturing cells either under anoxic conditions (Matthews et al., 1987) or by co-incubating with anti-oxidants or mitochondrial inhibitors (Yamauchi et al., 1989, Matsuda et al., 1991, Chang et al., 1992, Schulze-Osthoff et al., 1993, O'Donnell et al., 1995, Talley et al., 1995, Goossens et al., 1995), additional experiments were designed to assess the ability of TNFα to induce apoptosis in cells cultured under anoxic conditions. Such an approach allows us to determine whether the pro-apoptotic effect of this cytokine in neutrophils is mediated by (or dependent upon) the generation of reactive oxygen species or a capacity for aerobic respiration.

6.2 Results

6.2.1 Effect of C_6-ceramide and neutral sphingomyelinase on neutrophil apoptosis

In view of recent reports implicating TNFα-stimulated sphingomyelin hydrolysis and ceramide generation in mediating the pro-apoptotic activities of TNFα in leukaemic cell lines (Obeid et al., 1993, Jarvis et al., 1994), the effects of exogenous ceramide and sphingomyelinase on neutrophil apoptosis were examined. Incubation of neutrophils with a synthetic, cell-permeable C_6-ceramide analogue (10 μM) or a bacterial preparation of neutral sphingomyelinase (200 mU/ml), at concentrations known to induce apoptosis in other myeloid models (Obeid et al., 1993, Jarvis et al., 1994), did not induce apoptosis in these cells (figure 6.2.1). Identical data were obtained using 10 μM of the shorter chained synthetic C_2-ceramide (data not shown). In a separate series of experiments C_2- and C_6-ceramides were shown to mimic the effects of TNFα in inducing apoptosis in HL-60 cells as assessed by DNA fragmentation (MF Lawson, personal communication), confirming both the activity of the sphingolipids used in these studies and previously published data in this cell type (Jarvis et al., 1994). Hence, the capacity of exogenously applied ceramide to induce apoptosis in serum containing conditions appears to be highly cell type-specific.

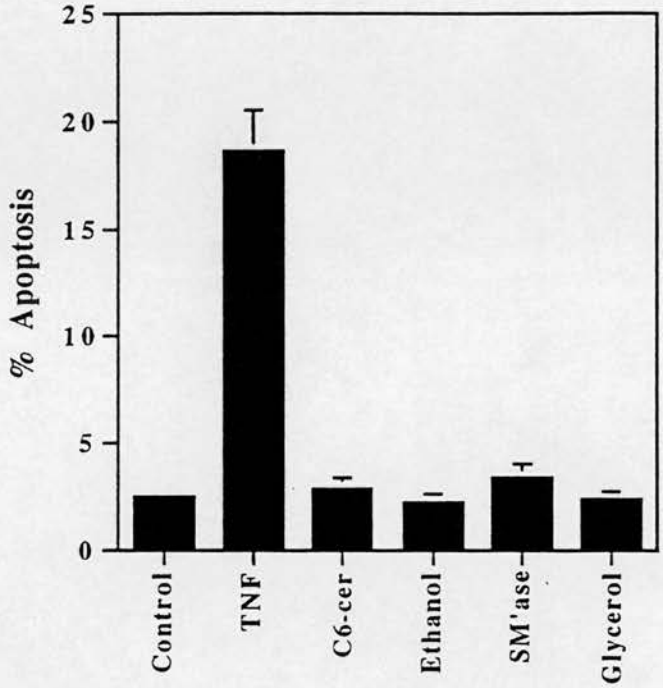


Figure 6.2.1 Effect of C_6-ceramide and neutral sphingomyelinase on neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were cultured for 6 hr either in serum-supplemented Iscove's MDM alone (control) or in identical medium containing : (final concentration) TNFα (12.5 ng/ml), C_6-ceramide (C_6-cer, 10μM), ethanol (0.1%, ceramide vehicle control), neutral sphingomyelinase (S'Mase, 200 mU/ml), or glycerol (0.27%, sphingomyelinase vehicle control). Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate. Where not shown, SEM values are <2%.

6.2.2 Effect of C_6-ceramide and neutral sphingomyelinase on fMLP-stimulated superoxide anion generation in neutrophils

Addition of the cell-permeable ceramide analogue C_6-ceramide (30 μM, 30 min, 37°C) produced no discernible direct or fMLP-priming effect on superoxide anion release; neutrophils from the same donor incubated in parallel with TNFα as a positive control exhibited classical augmentation of the superoxide anion response to fMLP (figure 6.2.2). The slight apparent effect of sphingomyelinase on superoxide generation observed was attributable to its vehicle (50% glycerol/PBS, 50 mM Tris-HCl, pH 7.5). It was concluded therefore that ceramide does not appear in isolation to mediate the priming effects of TNFα in human neutrophils, which agrees with data obtained by Yanaga and Watson (1995).

6.2.3 Effect of sphingosine on basal and TNFα-stimulated neutrophil apoptosis

In view of a previous report demonstrating that the pro-apoptotic effect of TNFα in human neutrophils could be mimicked by sphingosine (15 μM; Ohta et al., 1994), the effects of this sphingolipid on the rates of constitutive and TNFα-stimulated apoptosis was examined in these cells. In agreement with the previous study, the rate of apoptosis in neutrophils incubated for a 6 hr period in the presence of sphingosine (15 μM) was almost identical to that recorded for TNFα-stimulated cells (figure 6.2.3); the cells stimulated with sphingosine displayed classical morphological features of apoptosis. However the pro-apoptotic effect of sphingosine was entirely additive to that of TNFα, (figure 6.2.3), implying that these agents may utilize

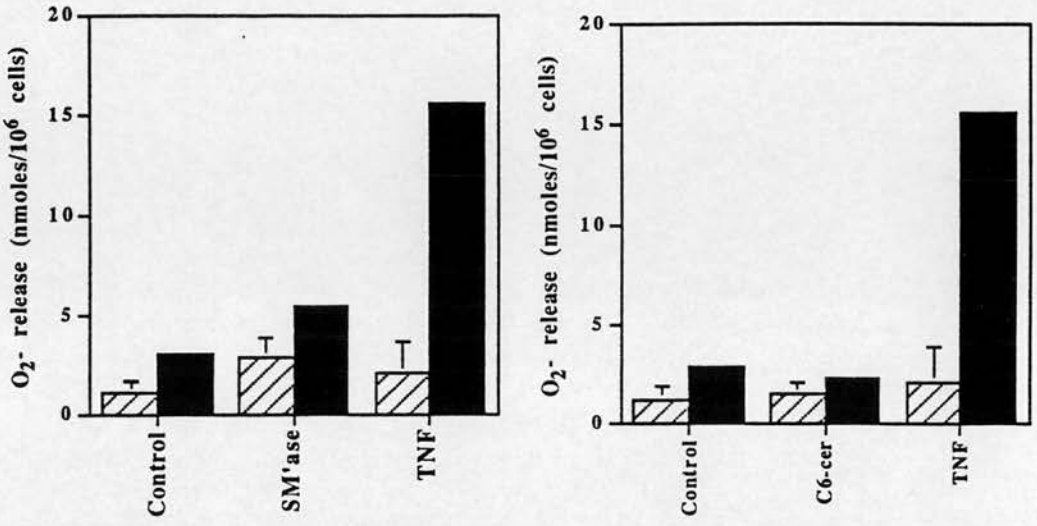


Figure 6.2.2 Effect of C_6-ceramide and neutral sphingomyelinase on fMLP-stimulated superoxide anion generation in neutrophils

Human neutrophils (10^6 in 90 µl PBS with $CaCl_2$ and $MgCl_2$) were incubated at 37°C with C_6-ceramide (C_6-cer, 30 µM), neutral sphingomyelinase (S'Mase, 200 mU/ml), TNFα (200 U/ml) or the appropriate vehicle for 30 min prior to the addition of pre-warmed cytochrome C (final concentration 1.2 mg/ml), and fMLP (final concentration 100 nM, filled bars) or buffer (hatched bars) to a final volume of 1 ml. SOD 375 U was incubated in one of each set of quadruplicate incubations. After 15 min the cells were pelleted at 4°C and the optical density of the supernatants determined by scanning spectrophotometry (550 nm); superoxide anion generation was calculated as described in the Materials and Methods section (2.5). Data represent mean ± SEM of 3 separate experiments, each performed in triplicate. (Experimental data provided by Dr AM Condliffe).

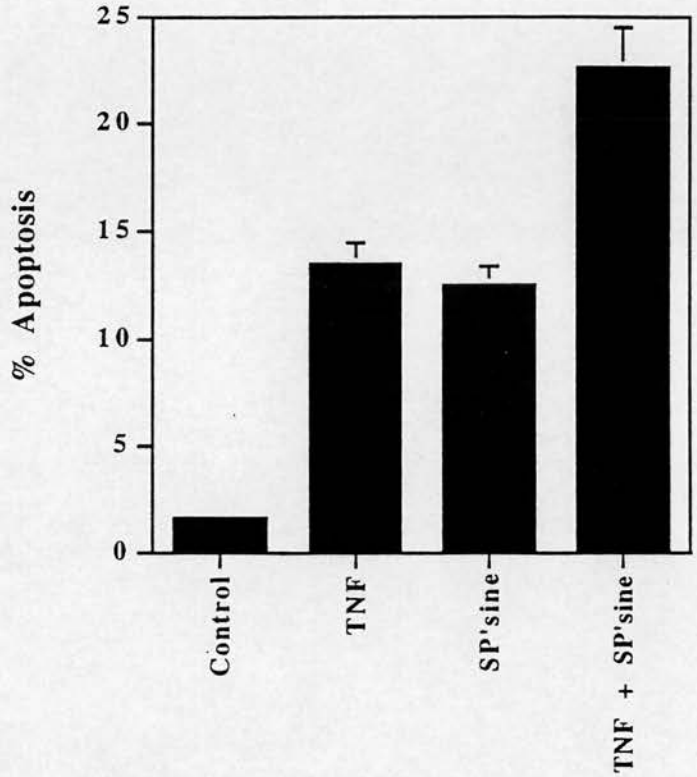


Figure 6.2.3 Effect of sphingosine on basal and TNFα-stimulated neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were incubated in serum-supplemented MDM alone (control) or in identical medium containing: (final concentration) TNFα (12.5 ng/ml), sphingosine (SP'sine, 15 μM) or TNFα (12.5 ng/ml) plus sphingosine (15 μM). Neutrophils were harvested following 6 hr in culture and apoptosis assessed morphologically. Data represent mean ± SEM of triplicate determinations in a single representative experiment of 3.

distinct rather than identical routes to signal cell death in human neutrophils. The pattern of effect of sphingosine was also very similar to that subsequently obtained using the PKC inhibitor Ro31-8220 (table 6.2.7) which may well explain the mechanistic basis of the sphingosine effect which would appear to work in parallel with the pro-apoptotic effects of TNFα.

6.2.4 The involvement of phosphoinositide 3-kinase in TNFα-stimulated neutrophil apoptosis

A number of very recent reports have proposed that agonist-stimulated activation of the phosphoinositide 3-kinase (PI3K) resulting in a phosphatidylinositol 3,4,5-trisphosphate (Ptd Ins (3,4,5) P_3)-dependent stimulation of PKB (Akt-1) is a powerful anti-apoptotic signal in certain cells (Hemmings, 1997, Toker and Cantley, 1997). To investigate the involvement of PI3K activity in mediating the pro-apoptotic effect of TNFα in neutrophils we examined the effect of the fungal metabolite wortmannin, which at the concentrations used in this study is a highly selective PI3K inhibitor (100 nM; Arcaro and Wyman, 1993), on basal and TNFα-stimulated neutrophil apoptosis. This agent, although highly unstable in an aqueous environment, causes rapid and irreversible inhibition of PI3K activity which is mediated via its interaction with the ATP binding site of the p110 subunits of both the PI3K isoforms found in neutrophils (Yano et al., 1993). This agent is non-toxic to neutrophils over 20 hr (trypan blue exclusion) and in parallel studies has been shown to completely ablate agonist-stimulated superoxide anion generation although

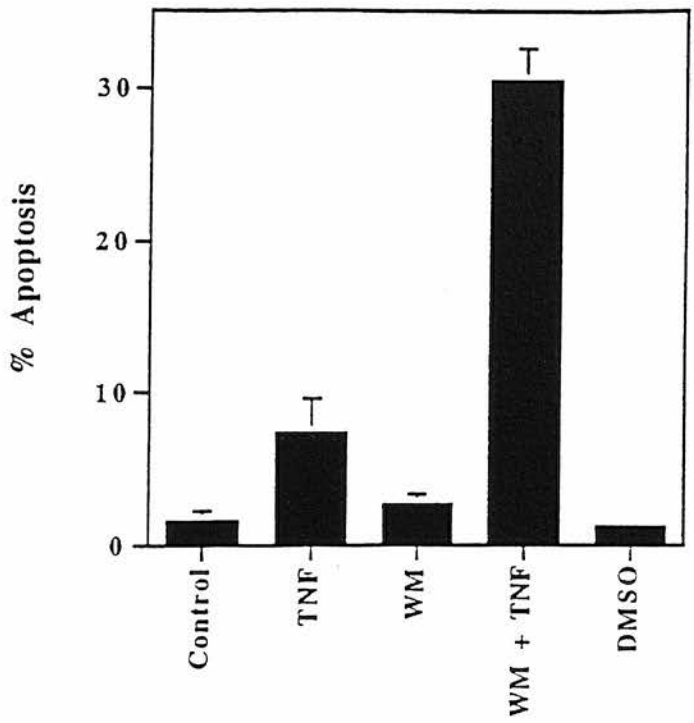


Figure 6.2.4 The involvement of PI3K in TNFα-stimulated neutrophil apoptosis
Human neutrophils ($10x10^6$/ml) were incubated for 6 hr in serum-supplemented Iscove's MDM in a final volume of 2 ml in bags prepared from heat-sealed Teflon film (Chemical Fabrics Corporation, New Hampshire, U.K.). Neutrophils were incubated for 6 hr either in Iscove's MDM alone (control) or in identical medium containing: (final concentration) TNFα (12.5 ng/ml), wortmannin (WM, 100 nM), TNFα (12.5 ng/ml) plus WM (100 nM), or DMSO vehicle (0.1%). WM was replenished hourly (10 µl, final concentration 50 nM) due to its instability in aqueous solution; controls and treatments were topped up simultaneously with medium or vehicle to control for the potential effects of cell resuspension, volume increase or cumulative DMSO effects. Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate.

does not affect PMA-induced NADPH activation (Laudanna et al., 1993; Dr ER Chilvers, personal communication).

Incubation of neutrophils with wortmannin (100 nM) alone had no effect on the basal rate of apoptosis at 6 hr but greatly augmented the ability of TNFα to induce apoptosis (figure 6.2.4). Augmentation of TNFα-stimulated apoptosis however was only observed when the concentration of wortmannin (100 nM) was replenished hourly (data not shown). Even using such a replenishment regime, wortmannin either on its own, or in the presence of TNFα, had no effect on cell viability or recovery. The relatively small pro-apoptotic effect of TNFα recorded in these experiments compared to data presented in chapter 3 reflects the combined effects of the higher cell concentrations used, and the requirement for a 30 min inhibitor pre-incubation period prior to TNFα addition (as discussed in Chapter 4).

6.2.5 The role of p38 MAP kinase in TNFα-mediated stimulation and LPS-mediated inhibition of apoptosis in neutrophils

The potential role of the p38 MAP kinase cascade in mediating the pro- and anti-apoptotic effects of TNFα and LPS respectively on neutrophils was examined by pre-incubation of cells with SB 203580 (20 μM, 30 min) an inhibitor of p38 Kinase (Lee et al., 1994). SB 203580 was originally discovered as an inhibitor of LPS-induced cytokine (IL-1 and TNFα) synthesis in THP-1 monocytes (IC_{50} 1 μM) (Lee et al., 1994) and subsequently to inhibit LPS-induced activation of MAPKAP

kinase-2, a physiological substrate for p38 kinase (IC_{50} 0.6 μM). SB 203580 has also been shown to prevent the phosphorylation of heat shock protein (hsp) 27 in response to IL-1, cellular stress and LPS in KB and PC12 cells (Cuenda et al., 1995) and block TNFα-induced transcription of the IL-6 gene in fibroblasts (Bayaert et al., 1996). Preliminary data obtained in collaboration with Drs R Plevin and A Paul (University of Strathclyde) and Professor P Cohen (University of Dundee) have demonstrated the capacity of SB 203580 to inhibit LPS and TNFα stimulated p38 activation and block LPS-induced DNA synthesis in RAW 264.7 macrophages (Paul et al., manuscript submitted).

Pre-incubation of neutrophils with SB 203580 (20 μM, 30 min) prior to stimulation with TNFα or LPS despite having no effect on basal apoptosis at these time points clearly potentiated TNFα-induced apoptosis (6 hr) (figure 6.2.5A) and suppressed LPS-mediated inhibition of apoptosis (20 hr) (figure 6.2.5B). These data implicate p38 MAP kinase activation in protection of neutrophils against apoptotic stimuli.

6.2.6 The role of the p42/44 MAP/ERK cascade in TNFα-mediated stimulation and LPS-mediated inhibition of apoptosis in neutrophils

The role of the "classical" p42/44 MAP/ERK cascade in mediating the pro- and anti-apoptotic effects of TNFα and LPS in neutrophils was examined by pre-incubation of cells with PD 098059 (50 μM, 30 min), a specific inhibitor of MEK-1 (Dudley et al., 1995), which lies immediately upstream of the p42/44 isoforms of MAPK. PD 098059

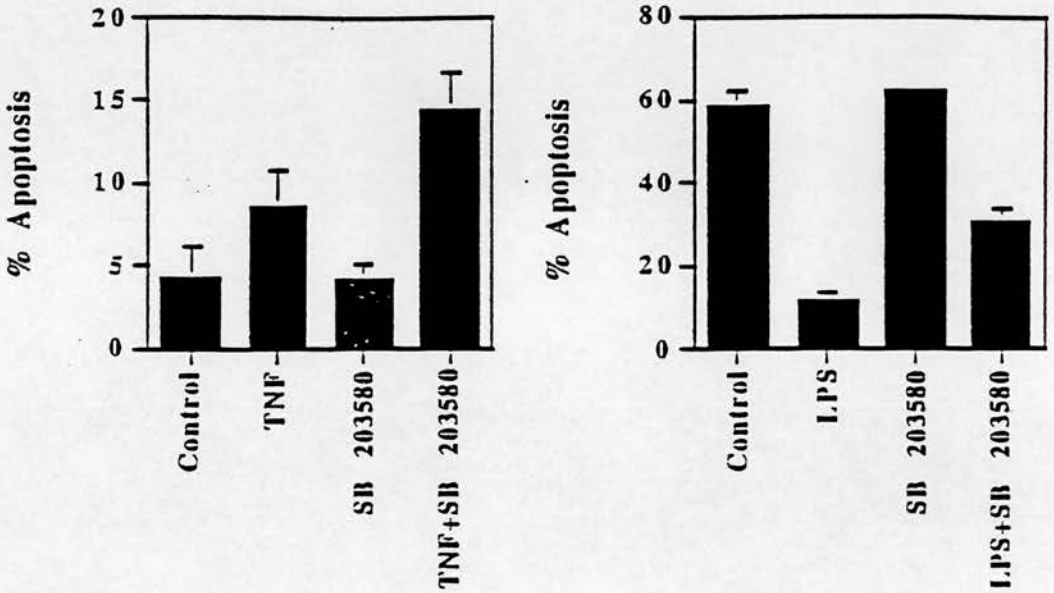


Figure 6.2.5 The role of p38 MAP kinase in TNFα-mediated stimulation and LPS-mediated inhibition of apoptosis in neutrophils

Human neutrophils ($5x10^6$/ml) were pre-incubated (37°C, 30 min) with 20 μM p38 MAP kinase inhibitor (SB 203580), or diluent control, in serum-supplemented Iscove's MDM for 30 min at 37°C prior to culture in the presence or absence of: 12.5 ng/ml TNFα for 6 hr (left hand panel), or 1 μg/ml LPS for 20 hr (right hand panel). Data represent mean ± SEM of 6 determinations from 2 independent experiments.

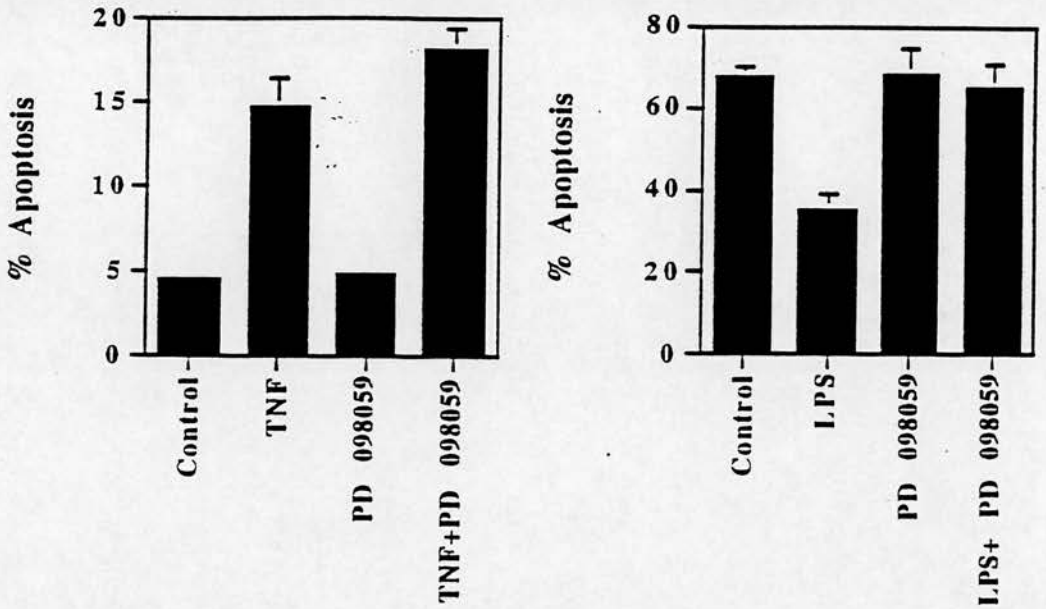


Figure 6.2.6 The role of the p42/44 MAP/ERK kinase cascade in TNFα-mediated stimulation and LPS-mediated inhibition of apoptosis in neutrophils

Human neutrophils ($5x10^6$/ml) were pre-incubated (37°C, 30 min) with 50 μM of the MEK-1 inhibitor (PD 098059), or diluent control, in serum-supplemented Iscove's MDM for 30 min at 37°C prior to culture in the presence or absence of: 12.5 ng/ml TNFα for 6 hr (left hand panel), or 1 μg/ml LPS for 20 hr (right hand panel). Data represent mean ± SEM of 6 determinations from 2 independent experiments.

has been demonstrated to prevent the activation of MEK-1 by Raf or MEKK *in vitro* (IC_{50} 2-7 μM; Alessi et al., 1995) but does not appear to compete at the ATP binding or MAPK binding site but inhibits in an unconventional manner through an allosteric mechanism which accounts for its high degree of selectivity for MEK-1 inhibition (Dudley et al., 1995). Furthermore, PD 098059 does not inhibit the biological function of MEK-1 once phosphorylated but instead prevents the activation and phosphorylation of MEK-1 (Dudley et al., 1995).

Pre-incubation of neutrophils with PD 098059 prior to stimulation with TNFα or LPS again potentiated the early TNFα-induced pro-apoptotic signal (6 hr) (figure 6.2.6A) but also completely attenuated the LPS-mediated inhibition of apoptosis (20 hr) (figure 6.2.6B) implying that stimulation of the p42/44 MAP/ERK cascade is a major pathway for LPS 'anti-apoptotic' signalling in neutrophils. These data are in agreement with a previous study performed in rat PC-12 cells where apoptosis was suppressed by NGF (which activates the p42/44 MAP/ERKs) and subsequent withdrawal of NGF from the culture medium led to p42/44 ERK inhibition which was accompanied by a stimulation of apoptosis (Xia et al., 1995).

6.2.7 The role of protein kinases in constitutive and TNFα-stimulated neutrophil apoptosis

In view of previous reports suggesting a role for protein tyrosine phosphorylation in the regulation of apoptosis in myeloid cells (Bergamaschi et al., 1993, Yousefi et al., 1994, Novogrodsky et al., 1994), we exploited known inhibitors of protein tyrosine kinases to assess the potential role of these enzymes in TNFα-induced neutrophil apoptosis. Neither genestein (50 μM), which has been reported to block GM-CSF-mediated inhibition of neutrophil apoptosis (Yousefi et al., 1994), or tyrphostin AG 1288 (10 μM), which has been shown to inhibit TNFα-induced cytotoxity in murine A9 fibroblasts (Novogrodsky et al., 1994), had any influence on TNFα-stimulated neutrophil apoptosis (table 6.2.7) indicating that an overall increase in protein tyrosine phosphorylation is not essential for TNFα-mediated cytotoxicity in these cells.

H-89, a specific inhibitor PKA (Combest et al., 1988, Chijiwa et al., 1990), has been used extensively in the neutrophil to examine the role of cAMP/PKA-mediated signalling events and has been recently demonstrated by our group to completely antagonise the survival effect in neutrophils of a range of prostanoid analogues and cAMP mimetics (Rossi et al., 1995). Likewise, it has been reported that TNFα inhibits cAMP accumulation in adherent neutrophils (Nathan and Sanchez, 1990) thereby suggesting the potential for TNFα to induce neutrophil apoptosis by reducing the concentration of this anti-apoptotic signalling molecule.

H-89 (100 μM), which should therefore enhance constitutive and/or TNFα-stimulated apoptosis, had no effect on either the basal rate of apoptosis at 6 hr or the early pro-apoptotic effect of TNFα (table 6.2.7). The conclusion that inhibiting cAMP is unlikely to explain the pro-apoptotic effect of TNFα is further supported by data presented in section 6.2.8 where pre-elevation of cAMP (using dibutyryl-cAMP) does not block TNFα cytotoxicity.

% Apoptosis	Control	Genistein	Tyr AG	H-89	Ro31-8220
Control	4.3 ± 1.2	2.1 ± 0.7	4.1 ± 1.2	6.1 ± 1.9	21.4 ± 5.6
TNFα	10.5 ± 1.2	8.2 ± 2.2	10.9 ± 2.1	10.9 ± 2.0	25.1 ± 4.5

Table 6.2.7 The role of protein kinases in constitutive and TNFα-stimulated neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were pre-incubated (37°C, 30 min) in serum-supplemented Iscove's MDM alone (control) or in identical medium containing: (final concentration) genistein (50 μM), tyrphostin AG 1288 (tyr AG, 10 μM), H-89, dihydrochloride (100 μM) or Ro31-8220 (1 μM) in the presence (closed bars) or absence (hatched bars) of 12.5 ng/ml TNFα. Neutrophils were harvested and assessed for morphological features of apoptosis. Data represent mean ± SEM of 5 separate experiments, each performed in triplicate.

In view of studies in U937 cells demonstrating an inhibition of TNFα-mediated apoptosis by the protein kinase C (PKC) activator PMA (Obeid et al., 1993), the role of PKC in TNFα-mediated neutrophil apoptosis was also assessed using Ro31-8220 (1 μM), a selective inhibitor of this enzyme. In agreement with a previous report (MKB Whyte, Ph D Thesis, 1993, Cousin et al., 1997), this inhibitor caused a marked increase in the rate of constitutive and TNFα-stimulated apoptosis (table 6.2.7) however, as with the previously detailed sphingosine data, it is difficult to draw mechanistic conclusions since these results most likely demonstrate activation of a parallel and complementary apoptotic pathway. The data however clearly do not support a role for TNFα-stimulated PKC in driving apoptosis and are very different to the results obtained by Obeid et al., (1993) in U937 cells.

It is important to re-emphasize that the relatively low pro-apoptotic effect of TNFα observed reflects the need in some of these experiments to pre-incubate neutrophils prior to exposure to TNFα which, as discussed in Chapter 4, severely abrogates the apoptotic efficacy of TNFα.

6.2.8 Involvement of cyclic nucleotides, nitric oxide and calcium-dependent signalling in TNFα-stimulated neutrophil apoptosis

Having failed to identify an obvious target kinase for TNFα-mediated apoptosis, a second panel of inhibitors were assayed to (i) provide additional data to support the premise that TNFα-mediated inhibition of cAMP accumulation was not critical to

this response, (ii) exclude a role for inducible nitric oxide synthase (iNOS) and Ca^{2+} in regulating TNFα-induced apoptosis (Bohlinger et al., 1995, Kim et al., 1997) and (iii) to explore in neutrophils, the report in U937 cells that protein phosphatase (PP) inhibitors augment TNFα-induced apoptosis (Wright et al., 1993).

Co-incubation with Ro-20-1724 (100 μM), a potent and selective inhibitor of cAMP-specific phosphodiesterase (PDE 4), which has previously been demonstrated to inhibit fMLP-stimulated superoxide anion generation in human neutrophils (Lad et al., 1985), caused a small and reproducible attenuation of TNFα-induced apoptosis (figure 6.2.8), however with n=4 experiments this failed to reach statistical significance. A similar but less pronounced trend was also observed with the stable and membrane permeable analogue of cAMP, dibutyryl cAMP (0.2 mM) (figure 6.2.8).

L-NIO HCl, a potent NO synthase inhibitor (Rees et al., 1990), and the Ca^{2+} ionophore A23187, that causes a profound suppression of neutrophil apoptosis at 20 hr (Whyte et al., 1993b), had no effect on basal or TNFα-stimulated apoptosis at 6 hr (figure 6.2.8). Finally, the role of protein phosphorylation in TNFα-mediated neutrophil apoptosis was investigated using calyculin A (20 nM), a cell-permeable serine-threonine phosphatase inhibitor, which potently inhibits both PP1 and PP2A at sub-nanomolar concentrations (Ishihara et al., 1989) and has been demonstrated to enhance fMLP-induced superoxide anion generation in human neutrophils (Djerdjouri et al., 1995). This also failed to influence TNFα-mediated neutrophil

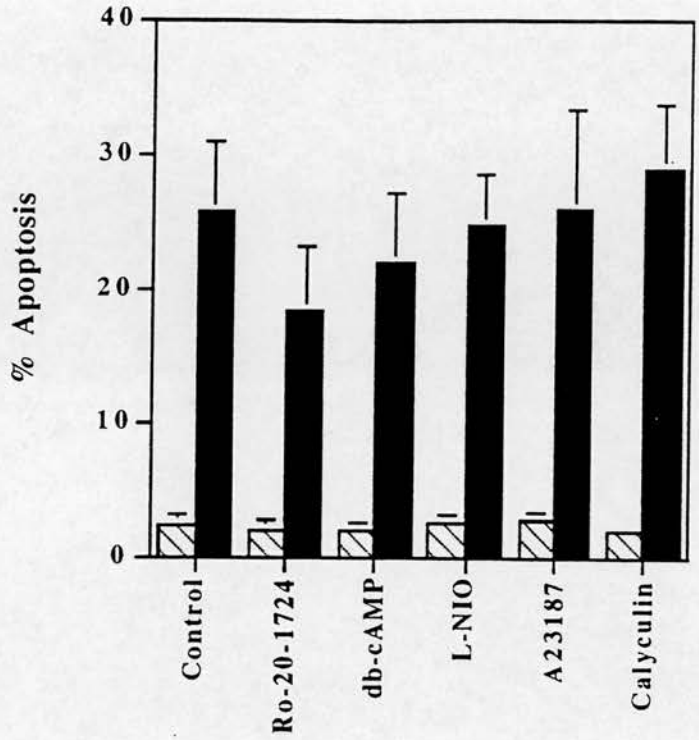


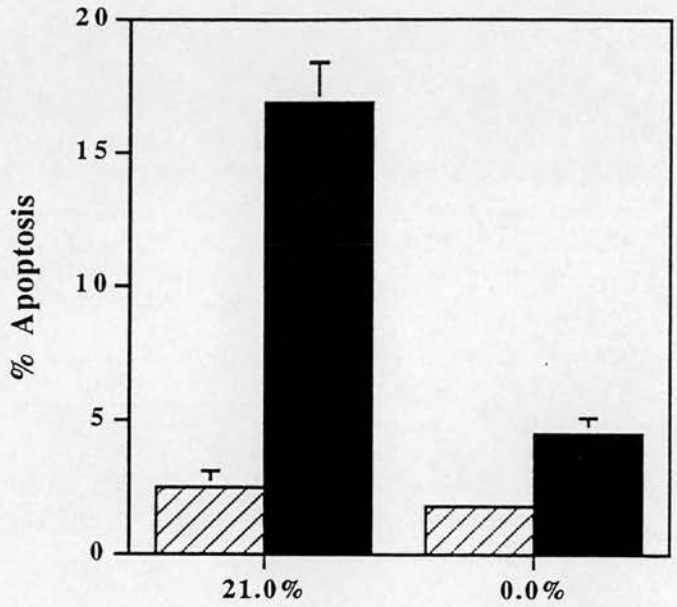
Figure 6.2.8 Involvement of specific signalling pathway in TNFα-stimulated neutrophil apoptosis

Human neutrophils ($5x10^6$/ml) were incubated for 6 hr in serum-supplemented Iscove's MDM alone (control) or in identical medium containing : final concentration) Ro-20-1724 (100 μM), dibutyryl-cAMP (db-cAMP, 0.2 mM), L-NIO. HCl (100 μM), A23187 calcium ionophore (0.1 μM) or calyculin A (20 nM) in the presence (closed bars) or absence (hatched bars) of TNFα (12.5 ng/ml). Neutrophils were harvested following 6 hr in culture and assessed for morphological features of apoptosis. Data represent mean ± SEM of 4 separate experiments, each performed in triplicate.

apoptosis (figure 6.2.8). These data suggest that, at least in the neutrophil, an overall increase in the level of serine-threonine protein phosphorylation is not an essential step in TNFα-mediated cytotoxicity and that early elevation of cAMP and Ca^{2+} fail to suppress the TNFα effect.

6.2.9 Effect of hypoxia on the pro-apoptotic effect of TNFα in neutrophils

To address whether the pro-apoptotic effect of TNFα in neutrophils involves or requires oxidative metabolism or reactive oxygen intermediates, the effect of extreme hypoxia on this response was examined. Neutrophils were cultured in the presence or absence of TNFα under normoxic (21% O_2) or 'anoxic' conditions using medium which had been fully deoxygenated before addition of TNFα. The pro-apoptotic effect of TNFα was greatly reduced under anoxic conditions (figure 6.2.9) implicating an essential role for molecular oxygen in the cytocidal effect of TNFα. In an initial set of experiments (data not shown) neutrophils were cultured under anoxic conditions but using medium which had not been deoxygenated prior to TNFα stimulation where full anoxia is not fully established until 30-60 min into the incubation period (KI Mecklenburgh, personal communication). Under these conditions TNFα induced neutrophil apoptosis at a rate not significantly different from its effect under normoxic conditions, indicating that the commitment of cells to undergo apoptosis in response to TNFα is likely to be extremely rapid, occurring within 30 min of exposure to this cytokine.



6.2.9 Effect of hypoxia on the pro-apoptotic effect of TNFα in neutrophils

Human neutrophils ($5x10^6$/ml) were cultured in serum-supplemented Iscove's MDM in the presence or absence of 12.5 ng/ml TNFα under conditions of normoxia (21% O_2) or anoxia (medium was fully deoxygenated prior to TNFα addition). Neutrophils were harvested following 6 hr in culture and apoptosis assessed morphologically. Data represent mean ± SEM of 3 separate experiments, each performed in triplicate; where not shown, SEM values are <2% of means (experimental data provided by Ms KI Mecklenburgh).

6.3 Discussion

In these studies we have sought to identify the intracellular mechanism(s) underlying the induction of neutrophil apoptosis by TNFα. In view of recent reports implicating TNFα-stimulated sphingomyelin hydrolysis and ceramide generation in mediating the pro-apoptotic effects of TNFα in leukaemic cell lines (Obeid et al., 1993, Jarvis et al., 1994) we wanted initially to explore the involvement of the ceramide/sphingomyelinase pathway in TNFα-induced apoptosis in neutrophils. Addition of a cell-permeable synthetic C_6-ceramide or a bacterial preparation of neutral sphingomyelinase, at concentrations that have been shown to induce rapid and highly efficient apoptosis in a number of other cell types (Obeid et al., 1993, Jarvis et al., 1994), was unable to induce apoptosis in neutrophils, or mimic TNFα-induced priming of fMLP-stimulated superoxide generation. In this context, it is interesting to note that bacterial endotoxin (LPS), which has a strong structural and functional resemblance to ceramide (Wright and Kolesnick, 1995), likewise inhibits rather than promotes neutrophil apoptosis even at early times (Lee et al., 1993). These data suggest that the sphingomyelin/ceramide pathway may not be the principal signalling route employed by TNFα in human neutrophils.

Despite such data, a number of other factors need to be considered prior to dismissing completely a role for TNFα-induced ceramide generation in the pro-apoptotic response in the neutrophil. Hence, in a collaborative study with Drs ML Barnard and JD Bell (Hammersmith Hospital, London) we have obtained evidence

on NMR spectroscopy for a relatively selective accumulation of phosphocholine (a by-product of ceramide generation from sphingomyelin) in TNFα-stimulated neutrophils (Nunn et al., 1996). Secondly, in agreement with a previous report of Ohta et al., (1994), our results indicate that sphingosine, an immediate downstream product of ceramide metabolism can both mimic and augment TNFα-induced apoptosis in neutrophils. Furthermore, in the study of Ohta and co-workers (1994), it is clear that TNFα can indeed stimulate both ceramide (68% increase, 5 min) and sphingosine (95% increase, 60 min) accumulation in neutrophils and from the kinetics of these two responses the authors propose that the increase in sphingosine observed following TNFα stimulation may have resulted from degradation of the newly-formed ceramide, moreover, the very high concentration of ceramide present in unstimulated cells again suggests that sphingosine rather than ceramide itself might function as an endogenous mediator of TNFα-induced apoptosis.

Hence, the role and function of the ceramide/sphingosine pathway in neutrophil apoptosis remains somewhat uncertain. If sphingosine is a genuine intermediate in this pathway, the lack of a similar response to sphingosine-1-phosphate (Ohta et al., 1994), the initial product of sphingosine catabolism, makes a direct effect of sphingosine-most likely the inhibition of PKC (Hannun et al., 1986)-the most probable mechanism of action.

Finally, a recent report by Higuchi and co-workers (1996) which demonstrated that addition of cell permeable ceramides was ineffective in inducing apoptosis, in the

myeloid cell line ML-1a (which is extremely sensitive to TNFα and cycloheximide-induced cell death), also showed that the calcium channel blocker SR33557 which inhibits the acidic (lysosomal) but not neutral sphingomyelinase (Jaffrezou et al., 1991, 1992) caused an approximately 50% inhibition of the pro-apoptotic effect of TNFα. Of note, this inhibition was partially reversed (by approximately 50%) following the addition of ceramides. From this study the authors concluded that ceramide generated by an acidic sphingomyelinase may be required but is insufficient alone for a TNFα-mediated death signal in these cells.

To explore the potential involvement of the PI3K pathway in TNFα-induced neutrophil apoptosis, we examined the effect of the fungal metabolite wortmannin, a highly specific PI3K inhibitor which abolishes agonist-stimulated superoxide anion generation in human neutrophils (Laudanna et al., 1993). In our experiments, wortmannin had no influence on the basal rate of apoptosis at 6 hr but augmented the cytocidal effect of TNFα. The suggestion that co-activation of PI3K may inhibit or retard the primary TNFα-stimulated apoptotic signalling pathway is supported by data showing that this enzyme plays a crucial role in NGF and PDGF-mediated survival in PC-12 cells (Yao and Cooper, 1995, 1996 Minshall et al., 1996) and several very recent reports proposing that agonist-stimulated activation of PI3K resulting in a Ptd Ins (3,4,5)P_3-dependent stimulation of PKB (Akt-1) is a powerful anti-apoptotic signal in certain cells (Toker and Cantley, 1997, Hemmings, 1997).

The inability of wortmannin to influence the basal rate of apoptosis in neutrophils suggests either a lack of involvement of this enzyme in regulating the rate of early constitutive apoptosis in these cells or inefficient enzyme inhibition perhaps due to involvement of the p101/p110 PI3K also found in neutrophils that is relatively insensitive to wortmannin. One particularly interesting aspect of the wortmannin-mediated augmentation of TNFα-induced apoptosis is that these data would support our earlier hypothesis that TNFα initiates dual survival and death signals reflected by the early induction and later inhibition of apoptosis by this cytokine. It is possible therefore that the late TNFR55-triggered survival signal is PI3K-mediated and that a much higher TNFα apoptotic response can be achieved if this synchronously triggered survival signal is abolished.

There has been a recent flurry of interest in mitogen-activated protein kinase (MAPK)-mediated signal transduction cascades which are conserved eukaryotic signalling pathways that convert receptor derived signals into a variety of cellular functions (for reviews see Davis , 1993, Marshall, 1995, Hunter, 1995). MAPKs are a family of dual-specificity protein kinases activated through threonine/tyrosine phosphorylation by MAPK kinases (MEKs), which are themselves activated by a series of MAPK kinase kinases (MEKKs; Blenis, 1993). Currently three distinct mammalian MAPKs, each with apparently unique signalling functions, have been identified. The ERK (extracellular signal-regulated kinase) group of MAPKs are activated by growth factors via *Ras*-dependent kinases (Raf and MEKK) that phosphorylate and activate the MAP/ERK kinases (MEK-1 and MEK-2) which in

turn activate the ERK-1 and ERK-2 MAPKs (p42 and p44 MAPKs; Derijard et al., 1995).

The JNK group of MAP kinases (also known as stress-activated protein kinases, SAPKs) activate the transcription factor c-Jun in response pro-inflammatory cytokines and cell exposure to several forms of environmental stress (Sanchez et al., 1994). JNK is activated by JNK kinase (Minden et al., 1994, Sanchez et al., 1994, Lin et al., 1995, Derijard et al., 1995,) which is itself regulated by phosphorylation by an upstream MEK kinase (MEKK; Lange-Carter et al., 1993); the MEKK-regulated JNK cascade is parallel to the Raf/ERK pathway.

The third group of MAPKs are mammalian homologues of HOG1 found in yeast and have been designated p38 MAP kinases on account of their molecular weight in murine cells (Han et al., 1994). In response to environmental stresses, mitogens, and pro-inflammatory cytokines, p38 MAPK is activated, and in turn stimulates MAPK-activated protein (MAPKAP) kinase-2 (Rouse et al., 1994, Freshney et al., 1994) which has been shown to be activated in human neutrophils upon stimulation by PMA or fMLP (Zu et al., 1996). The *in vivo* substrates of MAPKAP kinase-2 include heat shock protein (hsp) 27 and the transcription factors CREB and AFT1 (Cuenda et al., 1995, Tan et al., 1996). The phosphorylation of hsp27 is thought to stimulate the phosphorylation of actin, and thus help repair the actin microfilament network that becomes disrupted during cellular stress, thereby aiding cell survival (Lavioe et al., 1995).

A recent detailed study performed in rat PC-12 pheochromocytoma cells implicated the 'classical' Ras-dependent MAP kinase pathway (p42/44) and the cytokine-activated p38 MAP kinase pathway in the prevention and induction of apoptosis respectively (Xia et al., 1995). Moreover, it has also been demonstrated in myeloid cells that TNFα and LPS stimulate both the p42/p44 MAP/ERK and p38 kinase cascades (Yuo et al., 1993, Rafiee et al., 1995, Nick et al., 1996, Nahas et al., 1996). We were therefore keen to explore using selective pathway inhibitors the relative contribution of these MAPK cascades in TNFα-mediated induction and LPS-mediated inhibition of neutrophil apoptosis.

Both SB 203580, a specific inhibitor of p38 kinase (Cuenda et al., 1995, Lee et al., 1994), and PD 098059, an inhibitor of MEK-1 activation of p42/p44 MAPK (Alessi et al., 1995, Dudley et al., 1995), potentiated TNFα-mediated induction of apoptosis at 6 hr. Furthermore, PD 098059 completely reversed, and SB 203580 partially reversed, LPS-mediated inhibition of apoptosis at 20 hr. Taken together, these data implicate MAP/ERKs (p42/44) MAP kinases and to a lesser extent, p38 MAP kinase, in the protection of neutrophils against apoptotic stimuli.

The importance of the p42/p44 MAP/ERK kinases in cellular protection against apoptosis is consistent with the study performed in rat PC-12 cells, where apoptosis was suppressed by NGF which activates the p42/44 MAP kinases (Xia et al., 1995). The potential anti-apoptotic role of p38 MAP kinase was not identified in the studies however since withdrawal of NGF from the culture medium led to a sustained

activation of transfected p38 MAP kinase and JNK, and inhibition of p42/44 ERKs, which was accompanied by a stimulation of apoptosis. These data were interpreted as showing that apoptosis in NGF-differentiated PC-12 cells is regulated by the opposing actions of ERK and JNK-p38 MAP kinase pathways, however it is possible that activation of JNK was the dominant death trigger and that a true protective influence of p38 was missed. Although this issue remains to be resolved, it is clear that the dynamic balance that exists between the ERK and stress-activated JNK-p38 pathways may be crucial in determining whether a cell survives or undergoes apoptosis. Likewise, the capacity of PD098059 to protect neutrophils from the (adverse) effects of LPS on neutrophil survival, without affecting LPS priming (Drs M-H Sparagno and AG Rossi, personal communication), represents an important advance since it offers a potential route to enhance neutrophil clearance by inducing selective blockage of survival factor-induced cell longevity.

In view of previous studies implicating protein tyrosine phosphorylation in the regulation of apoptosis in myeloid cells (Bergamschi et al., 1993, Yousefi et al., Novogrodsky et al., 1994) we also sought to define the potential role of protein tyrosine kinases in TNFα-mediated neutrophil apoptosis again by exploiting well-established inhibitors of these enzymes. Neither genestein, which has been shown to inhibit both GM-CSF-mediated inhibition of neutrophil apoptosis (Yousefi et al., 1994) and TNFα-stimulated neutrophil adhesion to endothelial cell monolayers *in vitro* (McGregor et al., 1994), or tyrphostin AG 1288 which has been demonstrated to inhibit TNFα-induced cytotoxicity in murine A9 fibroblasts (Novogrodsky et al.,

1994), had any influence on TNFα-stimulated neutrophil apoptosis, implying that an overall increase in protein tyrosine phosphorylation is not an essential step in TNFα-mediated cytotoxicity in these cells.

In view of previous studies demonstrating inhibition of TNFα and C_2-ceramide-induced apoptosis in U937 cells by the PKC activator PMA and a similar inhibition of constitutive apoptosis in human neutrophils (Obeid et al., 1993, MKB Whyte, Ph D thesis, Cousin et al., 1997) the potential role of PKC activation in TNFα-induced neutrophil apoptosis was investigated. In agreement with the study by Cousin and colleagues (1997), the PKC inhibitor Ro-318220 induced a marked increase in the rate of neutrophil apoptosis. However, although the extent of TNFα-induced apoptosis was greater in the presence of Ro-318220, the ability of this agent to stimulate apoptosis *per se* makes it very difficult to assess the 'serial' role of PKC inhibition in TNFα-mediated neutrophil apoptosis. Indeed, the near-additivity of the TNFα and Ro-318220 pro-apoptotic effects makes it likely that PKC regulation of neutrophil apoptosis is independent of the TNFα-mediated death signal.

To explore the TNFα-mediated death signal in neutrophils in more detail, inhibitors of specific pathways implicated in TNFα-mediated cytotoxicity in other cell models were exploited. It is well documented that agonist-induced activation of human neutrophils is inhibited by cAMP-elevating agents such as the adenylyl cyclase activator forskolin, phosphodiesterase inhibitors, and receptor-directed prostaglandins (Darius et al., 1994, Pons et al., 1994, Armstrong et al., 1994).

Despite numerous reports demonstrating that elevation of cAMP promotes apoptosis in myeloid cells (e.g. McConkey et al., 1990, Lomo et al., 1995), a recent report from our laboratory demonstrated a paradoxical and profound inhibition of human neutrophil apoptosis by cAMP analogues and cAMP elevating agents which was completely reversed with H-89, a selective inhibitor of PKA (Rossi et al., 1995). In view of these data, and a previous report demonstrating that TNFα and CD11/CD18 (β2) integrins act synergistically to inhibit cAMP accumulation in human neutrophils (Nathan and Sanchez, 1990), our first strategy was to assess the potential for TNFα to induce apoptosis by inhibiting cAMP levels. However, elevation of intracellular cAMP with the cell-permeable mimetic, dibutyryl cAMP, or by inhibition of phosphodiesterase activity failed to ameliorate the pro-apoptotic effect of TNFα and likewise the PKA inhibitor H-89 did not enhance either basal or TNFα driven apoptosis.

Nitric oxide (NO) is a short-lived biological mediator which may be either pro-inflammatory or anti-inflammatory depending on its effective concentration at sites of inflammation (Barnes and Belvisi, 1993, Albina and Reichner, 1995, Brady and Poole-Wilson, 1995, Kubes, 1995, Loscalzo and Welsh, 1995, Moilanen and Vapaatalo, 1995, Barnes, 1996). A previous *in vivo* study has demonstrated that pharmacological doses of the NO donor sodium nitroprusside conferred complete protection against TNFα-induced hepatocyte apoptosis and secondary necrosis in D-galactosamine-sensitized mice (Bohlinger et al., 1995). In addition, a more recent study has demonstrated that the NO donor S-nitroso-N-acetylpenicillamine (SNAP)

protects rat hepatocytes from TNFα-stimulated apoptosis *in vitro* by inducing heat shock protein 70 expression (Kim et al., 1997). In view of these findings, the role of NO in TNFα-mediated neutrophil cytotoxicity was examined. While we have not performed a detailed biochemical analysis of the effects of iNOS expression or NO generation, incubation of neutrophils with an NO synthase inhibitor had no influence on basal or TNFα-mediated neutrophil apoptosis implying that NO generation does not function as a principal apoptotic signalling intermediate, or a protective factor.

The potential role of intracellular calcium ($[Ca^{2+}]i$) in TNFα-mediated neutrophil apoptosis was investigated using A23187, a calcium ionophore which has been previously reported to inhibit the constitutive rate of neutrophil apoptosis *in vitro* via transient elevations of $[Ca^{2+}]i$ (Whyte et al., 1993b). It was predicted therefore that if the apoptogenic effect of TNFα was mediated via inhibition of basal $[Ca^{2+}]i$ levels that A23187 would block its effect; however, A23187 had no influence on the pro-apoptotic effect of TNFα indicating that this cytokine signals neutrophil death via a calcium-independent route. This data is supported by a previous study from our laboratory which demonstrated that TNFα does not influence basal or agonist-induced $Ins(1,4,5)P_3$ or $[Ca^{2+}]i$ levels in neutrophils (Drs AM Condliffe and ER Chilvers, personal communication).

A previous study has demonstrated that okadaic acid and calyculin A, inhibitors of protein phosphatases, PP1 and PP2A, were synergistic with TNFα in the induction of apoptosis in wild type U937 cells and completely reversed the resistance of a TNFα-

resistant variant (U9-TR; Wright et al., 1993), indicating that protein phosphorylation may function as a critical factor determining sensitivity or resistance to TNFα-induced cytotoxicity in these cells. With this data in mind, we sought to explore the potential role of protein phosphorylation in TNFα-mediated neutrophil apoptosis. The cell-permeable serine-threonine inhibitor calyculin A had no influence on TNFα-mediated neutrophil apoptosis implying that, at least in the neutrophil, an overall increase in the levels of protein phosphorylation is certainly not an essential step in TNFα-mediated cytotoxicity

In certain cell lines, in particular the murine fibrosarcoma cell line L929, the cytotoxic effect of TNFα has been abrogated by culturing cells either under anoxic conditions (Matthews et al., 1987), or by co-incubating with anti-oxidants or mitochondrial inhibitors (Yamauchi et al., 1989, Matsuda et al., 1991, Chang et al., 1992, O'Donnell et al., 1995, Talley et al., 1995, Goossens et al., 1995). Moreover, cellular sensitivity or resistance to TNFα has been correlated with decreased or increased levels of superoxide dismutase respectively (Hirose et al., 1993). In addition, in L929 cells TNFα stimulation has been shown to result in a rapid and receptor-mediated rise in the intracellular levels of reactive oxygen species (Matthews et al., 1987, Larrick and Wright, 1992, Goossens et al., 1995), and ultrastructural changes in mitochondrial morphology that occur without any pronounced damage to other cellular organelles (Matthews et al., 1987, Schulze-Osthoff et al., 1992). These data lead to the proposal that TNFα induces cell death by causing mitochondrial dysfunction and upregulation of the production of

intracellular reactive oxygen intermediates. A recent publication from our laboratory reported that the constitutive rate of neutrophil apoptosis is inhibited when cells are cultured under hypoxic conditions (Hannah et al., 1995) and subsequently, the pro-apoptotic effects of E.Coli ingestion and phagocytosis of serum-opsonized Oil-red-O particles have been attributed to an oxidant-induced killing mechanism (Watson et al., 1996, Coxon et al., 1996).

To determine whether the pro-apoptotic effect of TNFα in neutrophils is mediated via generation of reactive oxygen species, the ability of this cytokine to mediate a death signal in cells cultured under anoxic conditions was investigated. In agreement with other studies implicating oxidative stress in TNFα-mediated cytotoxicity, cell culture under near anoxic conditions was the one intervention that resulted in a dramatic abrogation of the pro-apoptotic effect of TNFα. Of relevance, a recent detailed report presented data demonstrating that physical hypoxia (substitution of O_2 by nitrogen) and chemical hypoxia (induction by sodium cyanide) did not alter expression of the neutrophil TNFR55 (Scannel et al., 1995). This result eliminates the possibility that the inhibition of the pro-apoptotic effect observed in our studies was secondary to shedding or down-regulation of the TNFR55 receptor subtype. It is interesting to note however that a large number of agents that induce a dramatic respiratory burst response in the neutrophil and hence lead to the accumulation and release of substantial quantities of reactive oxygen intermediates do not trigger apoptosis, and wortmannin which blocks all activation of the NADPH oxidase as just shown does not in any way abrogate the TNFα killing effect. Hence further studies are needed to

determine whether TNFα–stimulated apoptosis in neutrophils can also be abrogated by other anti-oxidant strategies including mitochondrial inhibitors and uncouplers, and iron chelators in order to define the precise mechanism underlying the inhibition by hypoxia of the apoptotic effect of TNFα in these cells.

Chapter 7: Summary

Granulocyte apoptosis has been proposed as an important mechanism underlying the removal of redundant neutrophils from an inflammatory focus. The ability of many pro-inflammatory agents to impede this event *in vitro* suggests that such agents act not only in a priming and/or secretagogue capacity but also increase neutrophil longevity by delaying apoptosis. This thesis has examined whether this hypothesis holds true for all priming agents, in particular TNFα, which has been variably reported to either induce, delay, or have no effect on neutrophil apoptosis.

Incubation of neutrophils with various priming and activating agents for 20 hr either inhibited (LPS, LTB_4, GM-CSF, IP_6) or had no effect on (fMLP, PAF) the extent of neutrophil apoptosis. TNFα however, although causing a significant inhibition of neutrophil apoptosis at 20 hr, was pro-apoptotic at earlier times (8 hr) when the constitutive rate of apoptosis is still low; this effect was not observed with any of the other priming or activating agents examined at this time. Thus TNFα appears to act in a unique, bimodal manner to enhance the rate of constitutive neutrophil apoptosis at early times (6 hr) while inhibiting apoptosis at more delayed times (>12 hr).

The early pro-apoptotic effect of TNFα was confirmed by DNA fragmentation and propidium iodide binding and shown to be concentration-dependent with a near-identical EC_{50} value (2.8 ng/ml) to that observed for TNFα-induced priming of fMLP-stimulated superoxide anion generation. Moreover, the early cytocidal effect

of this cytokine was detectable within 2 hr, abolished by TNFα neutralizing antibody, and not associated with any change in cell viability or recovery.

While neutrophils prepared from different donors varied with respect to their susceptibility to TNFα-induced apoptosis, the intra-donor variation was very small with individuals showing a fairly stereotyped TNFα response across time. While the responsiveness of cells to the pro-apoptotic effect of TNFα was dramatically up-regulated following an oral glucocorticosteroid neutrophilia-induction protocol, short-term addition of the synthetic glucocorticoid, dexamethasone, partially inhibited TNFα-mediated cytotoxicity.

In optimizing our culture conditions, we observed that the pro-apoptotic effect of TNFα was greatest at lower cell densities, attenuated by even a short pre-incubation period prior to cytokine addition, and was independent of serum concentration.

Of relevance to the inflamed site, the ability of TNFα to accelerate apoptosis was completely lost if neutrophils were pre-treated with the priming agent PAF (1 μM, 5 min) or aged for 6 hr prior to TNFα addition. While the latter condition enhanced TNFα-mediated inhibition of apoptosis observed at 20 hr, the efficacy of the pro-apoptotic effect was not enhanced by co-culture with the anti-inflammatory cytokine IL-10.

TNFα-stimulated apoptosis was abolished by pre-incubation of neutrophils with selective blocking antibodies to both the TNFR55 (which contains the classical death-domain sequence and is entirely responsible for the TNFα priming effect in suspension neutrophils) and TNFR75 receptor subtypes. Moreover, TNFR55-selective agonist mutant proteins (E146K, R32W-S86T) induced neutrophil apoptosis but with a potency 14-fold lower than wild type TNFα, while a TNFR75-selective mutant (D143F) did not induce apoptosis. These data indicate that TNFα has the ability, apparently unique to this priming agent, to induce apoptosis in human neutrophils at early time points via a mechanism whereby the TNFR75 acts to facilitate and permit TNFR55-mediated induction of cell death.

The pro-apoptotic effect of TNFα was not mimicked by exogenous addition of cell-permeable synthetic ceramides or neutral sphingomyelinase, suggesting that a sphingomyelin/ceramide triggered death pathway as observed in certain leukaemic cell lines may not be the principal signalling route employed by TNFα in human neutrophils. Inhibition of p38 MAP kinase and MAPKK-1 potentiated the pro-apoptotic effect of TNFα, while suppressing the protective effect of LPS at a later (20 hr) time, implicating the p38 and p42/44 MAP kinases in the protection of neutrophils against apoptotic stimuli. Additional pharmacological inhibitor-based studies excluded a major role for cAMP-PKA, PKC, PTK and NO/cGMP/PKG pathways in mediating the cytotoxic effect of TNFα.

Finally, although human neutrophils appear quite resistant to oxidant-induced killing (e.g. by H_2O_2, hyperoxia or NADPH oxidase activation), and wortmannin which ablates superoxide anion generation in these cells actually augments TNFα-induced killing, culture of neutrophils under hypoxic conditions was the one intervention that resulted in a dramatic abrogation of the pro-apoptotic effect of TNFα. This latter finding implicates either an oxygen-dependent step in TNFα-mediated neutrophil apoptosis or the capacity for the survival response triggered by anoxia to override the TNFα killing effect.

The recognition that neutrophil apoptosis can be stimulated by TNFα and that the priming and apoptotic effects of this cytokine can be dissociated may help in the design of more rational and selective anti-inflammatory therapies and underlines further the potential benefits of local TNFα generation at an inflamed site.

Publications arising from this thesis

Full publications

Murray J, Condliffe AM, Haslett C, Chilvers ER: Wortmannin enhances tumour necrosis factorα-stimulated neutrophil apoptosis. Biochem Soc Trans 224: 80S, 1996

Nunn AVW, Barnard ML, Bhakoo K, **Murray J**, Chilvers ER, Bell JD: Characterisation of secondary metabolites associated with neutrophil apoptosis. FEBS Letts 392: 295, 1996

Ward C, **Murray J**, Bruce L, Farrow S, Chilvers ER, Hannah S, Haslett C, Rossi AG: Interleukin-10 does not directly affect the constitutive rate of human neutrophil or eosinophil apoptosis. Biochem Soc Trans 25: 245S, 1997

Murray J, Barbara JAJ, Dunkley SA, Lopez AF, Van Ostade X, Condliffe AM, Dranssfield I, Haslett C, Chilvers ER: Regulation of neutrophil apoptosis by tumour necrosis factor-α: Requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. Blood 90: 2772, 1997

Chilvers ER, Rossi AG, **Murray J**, Haslett C: Regulation of granulocyte apoptosis and implications for anti-inflammatory therapy. Thorax, 1998, *in press*

Abstracts

Chilvers ER, **Murray J**, Condliffe AM, Haslett C: Stimulation of neutrophil apoptosis by TNFα: Differential effect compared to other priming agents. J Cell Biochem supp 19B: 269, 1995

Murray J, Haslett C, Chilvers ER: Wortmannin, a phosphatidylinositol 3-hydroxykinase (PI3K) inhibitor, augments tumour necrosis factor (TNFα) stimulated neutrophil apoptosis. Am J Respir Crit Care Med 151: A456, 1995

Murray J, Kitchen E, Chilvers ER: The pro-apoptotic effect of TNFα in human neutrophils is mediated via the TNF CD120b (p75) receptor. Br J Pharmacol 118: 8P, 1996

Murray J, Mecklenburgh KI, Ward C, Rossi AG, Haslett C, Chilvers ER: Intracellular signaling mechanisms in TNFα-stimulated apoptosis in human neutrophils (*manuscript in preparation*).

Abstracts and presentations

Murray J, Condliffe AM, Haslett C, Chilvers ER: Induction of neutrophil apoptosis by TNFα: Differential effect compared to other priming agents. Clin Sci. 88: 28P, 1995

Chilvers ER, **Murray J**, Condliffe AM, Haslett C: Stimulation of neutrophil apoptosis by TNFα: Differential effect compared to other priming agents. J Cell Biochem supp 19B: 269, 1995

Murray J, Haslett C, Chilvers ER: Wortmannin, a phosphatidylinositol 3-hydroxykinase (PI3K) inhibitor, augments tumour necrosis factor (TNFα) stimulated neutrophil apoptosis. Am J Respir Crit Care Med 151: A456, 1995

Murray J, Kitchen E, Chilvers ER: The pro-apoptotic effect of TNFα in human neutrophils is mediated via the TNF CD120b (p75) receptor. Br J Pharmacol 118: 8P, 1996

Mecklenburgh KI, **Murray J,** Haslett C, Chilvers ER: Hypoxia inhibits tumour necrosis factor-α-stimulated apoptosis in human neutrophils. Am J Respir Crit Care Med, 155: A446, 1997

Dunkley SA, Murray J, Haslett C, Chilvers ER: Fas-mediated regulation of human neutrophil apoptosis. Am J Crit Care Med, 1998, *in press*

Murray J, Rossi AG, Haslett C, Barbara JAJ, Chilvers ER: The pro-apoptotic effect of TNFα in human neutrophils requires both receptor (TNFR55 and TNFR75) subtypes. Am J Respir Crit Care Med, 1998, *in press*

References

Abe Y, Osuka Y, Nadata T, Kashu Y, Kimura S: The functional role of 55- and 75-kDa tumour necrosis factor receptors in human polymorphonuclear cells in vitro. Cytokine 7: 39, 1995

Aderka D, Engelmann H, Maor Y, Brakebusch C, Wallach D: stabilization of the bio-activity of tumour necrosis factor (TNF) by its soluble receptors. J Exp Med 175: 323, 1992

Afford SC, Pongracz J, Stockley RA, Crocker J, Burnett D: The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. J Biol Chem 267: 21612, 1992

Aggarwal BB, Raj KP (eds): Human cytokines: Their role in disease and therapy. Blackwell Science, USA, 1995

Alabaster VA, Keir RF, Parry MJ, De Souza RN: UK-74,505 a novel and selective PAF antagonist, exhibits potent and long lasting activity in vivo. Agents Actions 34: S221, 1991

Albeda SM, Smith CW, Ward PA: Adhesion molecules and inflammatory injury. FASEB J 8: 504, 1994

Albina JE, Reichner JS: Inhaled nitric oxide therapy. Mayo Clin. Proc 70: 247, 1995

Albrich JM, Gilbaugh JH, Callahan KB, Hurst JK: Effects of the putative neutrophil-generated toxin, hypochlorous acid, on membrane permeability and transport systems of *Escherischia coli*. J Clin Invest 78: 177, 1986

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270: 27489, 1995

Alison MR, Sarraf CE: Apoptosis: a gene-directed form of cell death. J Roy Coll Phys Lond 26: 25, 1992

Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J: Human ICE/CED-3 protease nomenclature. Cell 87: 171, 1996

Arcaro A, Wymann MP: Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J 296: 297, 1993

Arends MJ, Morris RG, Wyllie AH: Apoptosis: the role of the endonuclease. Am J Path 136: 593, 1990

Armitage RJ, Fanslow WC, Strockbine L, Sato TA, Clifford KN, Macduff BM, Anderson, DM, Gimpel SD, Davies-Smith T, Maliszewski CR: Molecular and biological characterization of a murine ligand for CD40. Nature 357: 80, 1992

Armstrong RA, Talpain E: Comparison of the prostaglandin E (EP) receptor of human neutrophils and HL-60 cells differentiated with DMSO. Prostaglandins 48: 221, 1994

Atkinson YH, Marasco WA, Lopez AF, Vadas MA: Recombinant tumor necrosis factor-α: regulation of N-formylmethionyl-leucylphenyl-alanine receptor affinity and function on human neutrophils. J Clin Invest 81: 759, 1988

Babior BM, Kipnes RS, Curnutte JT: Biological defense mechanisms: the production by leukocytes of superoxide, a potential bactericidal agent. J Clin Invest 52: 741, 1973

Baggiolini M, Walz A, Kunkel SL: Neutrophil-activating peptide/interleukin 8, a novel cytokine that activates neutrophils. J Clin Invest 84: 1045, 1989

Bainton DF, Miller LJ, Kishimoto TK, Springer TA: Leukocyte adhesion receptors are stored in peroxidase-negative granules of human neutrophils. J Exp Med 166: 1641, 1987

Baldridge CW, Gerard RW: The extra respiration of phagocytosis. Am J Physiol 103: 235, 1933

Barbara JAJ, Smith WB, Gamble JR, Van Ostade X, Vandenabeele P, Tavernier J, Fiers W, Vadas MA, Lopez AF: Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants. EMBO J 13: 843, 1994

Barnes PJ, Belvisi MJ: Nitric oxide and lung disease. Thorax: 48 1034, 1993

Barnes PJ: NO or NO in asthma? Thorax: 51 218, 1996

Bazzoni F, Cassatella MA, Rossi F, Ceska M, Dewald B, Baggiolini M: Phagocytosing neutrophils produce and release high amounts of the neutrophil-activating peptide 1/interleukin-8. J Exp Med 173: 771, 1991

Beaulieu AD, Paquin R, Rathanaswami P, McColl SR: Nuclear signalling in human neutrophils: stimulation of RNA synthesis is a response to a limited number of proinflammatory agonists. J Biol Chem 267: 426, 1992

Beg AA, Baltimore D: An essential role for NFκB in preventing TNF-α-induced cell death. Science 274: 782, 1996

Begley CG, Lopez AF, Nicola NA: Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in vitro: a rapid and sensitive microassay for colony-stimulating factors. Blood 68: 162, 1986

Behr J, Maier K, Krombach F, Adelmann-Grill BC: Pathogenic significance of reactive oxygen species in diffuse fibrosing alveolitis. Am Rev Respir Dis 144: 146, 1991

Belka C, Wiegmann K, Adam D, Holland R, Neuloh M, Herrmann F, Kronke M, Brach MA: Tumor necrosis factor (TNF)-α activates c-raf-1 kinase via the p55 TNF receptor engaging neutral sphingomyelinase. EMBO J 14: 1156, 1995

Bender JG, McPhail LC, Van Epps DE: Exposure of human neutrophils to chemotactic factors potentiates activation of the respiratory burst enzyme. J Immunol 130: 2316, 1983

Bergamaschi G, Rosti V, Danova M, Ponchio L, Lucotti C, Cazzola M: Inhibitors of tyrosine phosphorylation induce apoptosis in human leukemic cell lines. Leukemia 7: 2012, 1993

Berger M, Medof ME: Increased expression of complement decay-accelerating factor during activation of human neutrophils. J Clin Invest 79: 214, 1987

Bernofsky C: Nucleotide chloramines and neutrophil-mediated cytotoxicity. FASEB J 5: 295, 1991

Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YC, Mathison J, Ulevitch R, Cerami A: Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature 316: 552, 1985

Beutler B: Tumour Necrosis Factors: The Molecules and Their Emerging Role in Medicine. Raven Press Ltd, New York, 1992

Beutler B, van Huffel C: Unraveling function in the TNFα ligand and receptor families. Science 264: 667, 1994

Bevilacqua MP, Nelson RM: Selectins. J Clin Invest 91: 379, 1993

Bevilacqua MP, Pober JS, Majeau GR, Fiers W, Cotran RS, Gimbrone MA: Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. Proc Natl Acad Sci USA 83: 4533, 1986

Beyaert R, Fiers W: Molecular mechanisms of tumor necrosis factor-induced cytotoxicity: What we understand and what we do not. FEBS Lett 340: 9, 1994

Beyaert R, Cuenda A, Vanden Bergh W, Plaisance S, Lee JC, Haegeman G, Cohen P, Fiers W: The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis response to tumour necrosis factor. EMBO J 15: 1914, 1996

Biffl WL, Moore EE, Moore FA, Barnett CC: Interleukin-6 suppression of neutrophil apoptosis is neutrophil concentration dependent. J Leukoc Biol 58: 582, 1995

Bigda J, Beletsky I, Brakebusch C, Varfolomeeu Y, Engelmann H, Bigda J, Holfmann H, Wallach D: Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. J Exp Med 180: 445, 1994

Blenis J: Signal transduction via the MAP kinases: Proceed at your own RSK. Proc Natl Acad Sci USA 90: 5889, 1993

Bodmer JL, Burns K, Schneider P, Hofmann K, Steiner V, Thome M, Bornand T, Hahne M, Schroter M, Becker K, Wilson A, French LE, Browning JL, MacDonald HR, Tschopp J: TRAMP, a novel apoptosis-mediating receptor with sequence

homology to tumor necrosis factor receptor 1 and Fas (Apo-1/CD95). Immunity 6: 79, 1997

Bohlinger I, Leist M, Barsig J, Uhlig S, Tiegs G, Wendel A: Interleukin-1 and nitric oxide protect against tumour necrosis alpha-induced liver injury through distinct pathways. Hepatology 22: 1829, 1995

Boldin MP, Varfolomeev EE, Pancer Z, Mett IL, Camonis JH, Wallach D: A novel protein that interacts with the death domain of Ras/APO1 contains a sequence motif related to the death domain. J Biol Chem 270: 7795, 1995

Boldin MP, Goncharov TM, Goltsev YV, Wallach D: Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 85: 803, 1996

Borregaard N, Tauber AI: Subcellular localization of the neutrophil NADPH oxidase: *b*-cytochrome and associated flavoprotein. J Biol Chem 259: 47, 1984

Borregaard N, Lollike K, Kjeldsen L, Sengelov H, Bastholme L, Neilsen MH, Bainton D: Human neutrophil granules and secretory vesicles. Eur J Haematol 51: 187, 1993

Brach MA, de Vos S, Gruss H-J, Herrman F: Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. Blood 80: 2920, 1992

Brady AJ, Poole-Wilson PA: Circulatory failure in septic shock. Nitric oxide: too much of a good thing. Br. Heart J 70: 103, 1995

Brenner DA, O'Hara M, Angel P, Chojkier M, Karin M: Prolonged activation of jun and collagenase genes by tumor necrosis factor-alpha. Nature 337: 661, 1989

Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunzicker W, Lesslauer W, Loetscher H: Identification of two types of tumour necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci USA 87: 3127, 1990

Browning JL, Androlewicz MJ, Ware CF: Lymphotoxin and an associated 33-kDa glycoprotein are expressed on the surface of an activated human T cell hybridoma. J Immunol 147: 1230, 1991

Calafat J, Kuijpers TW, Janssen H, Borregaard N, Verhoeven, AJ, Roos D: Evidence for small intracellular vesicles in human blood phagocytes containing b_{558} and the adhesion molecule CD11/CD18. Blood 81: 3122, 1993

Camerini D, Walz G, Loenen WA, Borst J, Seed B: The T cell activation antigen CD27 is a member of the nerve growth factor/tumor necrosis factor receptor gene family. J Immunol 147: 3165, 1991

Cannistra SA, Griffin JD: Regulation of the production and function of granulocytes and monocytes. Semin Hematol 25: 173, 1988

Carlos TM, Harlan JM: Leukocyte-endothelial molecules. Blood 84: 2068, 1994

Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B: An endotoxin-induced serum factor that causes necrosis of tumours. Proc Natl Acad Sci USA 72: 3666, 1975

Cassatella MA, Meda L, Bonora S, Ceska M, Constantin G: IL-10 inhibits the release of pro-inflammatory cytokines from polymorphonuclear leukocytes. Evidence for an autocrine role of tumor necrosis factor and IL-1β in mediating the production of IL-8 triggered by lipopolysaccharide. J Exp Med 178: 2207, 1993

Chang DJ, Ringold GM, Hellar RA: Hyperoxide metabolism in mammalian organs. Physiol Rev 59: 527, 1979

Cheng G, Cleary AM, Ye Z-S, Hong DI, Lederman S, Baltimore D: Involvement of CRAF1, a relative of TRAF in CD40 signaling. Science 267: 1494, 1995

Chilton FH, Ellis JM, Olson SC, Wykle RL: 1-*0*-Alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine: a common source of platelet-activating factor and arachidonate in human polymorphonuclear leukocytes. J Biol Chem 259: 12014, 1984

Chinnaiyan AM, O'Rourke K, Tewari M, Dixit VM: FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81: 505, 1995

Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, Dixit VM: FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. J Biol Chem 271: 4961, 1996a

Chinnaiyan AM, Yu GL, Lyons RH, Garg M, Duan M, Duan DR, Xing L, Gentz R, Ni J, Dixit VM: Signal transduction by DR3, a death domain-containing receptor related to TNFR-1 and CD95. Science 274: 990, 1996b

Cicco N, Lindemann A, Content J, Vandenbussche P, Lubbert M, Gauss J, Mertelsmann R, Hermann F: Inducible production of interleukin-6 by human polymorphonuclear neutrophils: role of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-alpha. Blood 75: 2049, 1990

Clancy RM, Dahinden CA, Hugli TE: Arachidonate metabolism by human polymorphonuclear leukocytes stimulated by *N*-formyl-Met-Leu-Phe or complement component C5a is independent of phospholipase activation. Proc Natl Acad Sci USA 80: 7200, 1983

Coley WB: late results of the treatment of inoperable sarcoma by the mixed toxins of erysipelas and *Bacillus prodigiosus*. Am J Med Sci 131: 375, 1906

Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A: Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 80: 2012, 1992

Condliffe AM, Chilvers ER, Haslett C, Dransfield I: Priming differentially regulates neutrophil adhesion molecule expression/function. Immunology 89: 105, 1996

Cousin JM, Haslett C, Rossi AG: Regulation of granulocyte apoptosis by PKC inhibition and elevation of $[Ca^{2+}]i$. Biochem Soc Trans 25: 243S, 1997

Cox G, Gauldie J, Jordana M: Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. Am J Respir Mol Cell Biol 7: 507, 1992

Cox G, Crossley J, Xing Z: Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. Am J Respir Cell Mol Biol 12: 232, 1995a

Cox G: Glucocorticoid treatment inhibits apoptosis in human neutrophils. Separation of survival and activation outcomes. J Immunol 154: 4710, 1995

Cox G: IL-10 enhances resolution of pulmonary inflammation in vivo by promoting apoptosis of neutrophils. Am J Physiol 271: L566, 1996

Coxon A, Rieu P, Barkalow FJ, Askari SA, Sharpe AH, von Adrian UH, Arnaout MA, Mayadas TN: A novel role for the β2 integrin CD11/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation. Immunity 5: 653, 1996

Cronstein BN, Weissmann G: The adhesion molecules of inflammation. Arthritis Rheum 36: 147, 1993

Crowe PD, VanArsdale TL, Walter BN, Ware CF, Hession C, Ehrenfels B, Browning JL, Din WS, Goodwin RG, Smith CA: A lymphotoxin-beta-specific receptor. Science 264: 707, 1994

Cuenda A, Rouse J, Daza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC: SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett 364: 229, 1995

Dale DC, Fauci AS, Guerry DP IV, Wolff SM: Comparisons of agents producing a neutrophilic leucocytosis in man. J Clin Invest 56: 808, 1975

Daniels RH, Finnen MJ, Hill ME, Lackie JM: Recombinant human monocyte IL-8 primes NADPH-oxidase and phospholipase A_2 activation in human neutrophils. Immunology 75: 157, 1992

Darius H, Michael-Hepp J, Thierauch KH, Fisch A: Inhibition of human platelets and polymorphonuclear neutrophils by the potent and metabolically stable prostaglandin D2 analogue ZK 118.182. Eur J Pharmacol 258: 207, 1994

Davis RJ: The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 268: 14553, 1993

Della Bianca V, Grzeskowiak M, Dusi S, Rossi F: Transmembrane signaling pathways involved in phagocytosis and associated activation of NADPH oxidase mediated by FcγRs in human neutrophils. J Leukoc Biol 53: 427, 1993

Derijard D, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ, Davis RJ: Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms. Science 267:682, 1995

De Togni P, Goellner J, Ruddle NH et al.,: Abnormal development of peripheral lymphoid organs in mice deficient in lymphotoxin. Science 264: 703, 1994

DeWaal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE: Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 174: 1209, 1991

Dhabhar FS, Miller AH, Stein M, McEwen BS, Spencer RL: Diurnal and acute stress-induced changes in distribution of peripheral blood leukocyte subpopulations. Brain Behav Immunity 8: 66, 1994

DiDonato JA, Mercurio F, Rosette C, Wu-Li J, Suyang H, Ghosh S, Karin M: Mapping of the inducible IκB phosphorylation sites that signal its ubiquination and degradation. Mol Cell Biol 16: 1295, 1996

DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M: A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature 388: 548, 1997

DiPersio JF, Billing P, Williams R, Gasson JC: Human granulocyte-macrophage colony-stimulating factor and other cytokines prime neutrophils for enhanced arachidonic acid release and leukotriene B_4 synthesis. J Immunol 140: 4315, 1988

Djerdjouri B, Combadiere C, Pedruzzi E, Hakim J, Perianin A: Contrasting effects of calyculin A and okadaic acid on the respiratory burst of human neutrophils. Eur J Pharmacol 288: 193, 1995

Doerfler ME, Danner RL, Shelhamer JH, Parrillo JE: Bacterial lipopolysaccharide primes human neutrophils for enhanced production of leukotriene B_4. J Clin Invest 83: 970, 1989

Doerfler ME, Weiss J, Clark JD, Elsbach P: Bacterial lipopolysaccharide primes human neutrophils for enhanced release of arachidonic acid and causes phosphorylation of an 85-kD cytosolic phospholipase A_2. J Clin Invest 93: 1583, 1994

Doerschuk CM, Markos J, Coxon HO, English D, Hogg JC: Quantification of neutrophil migration in acute bacterial pneumonia in rabbits. J Appl Physiol 77: 2593, 1994

Donnelly SC, Haslett C: Cellular mechanisms of acute lung injury: implications for future treatment in the adult respiratory distress syndrome. Thorax 47: 260, 1992

Dornay BG, Reddy SAG, Aggarwal BB: Physical and functional association of a serine-threonine protein kinase to the cytoplasmic domain of the p80 form of the human tumor necrosis factor receptor in human histiocytic lymphoma V-937 cells. J Biol Chem 269: 19687, 1994

Downey GP, Worthen GS, Henson PM, Hyde DM: Neutrophil sequestration and migration in response to local instillation of C5a in the lung: Capillary localization and migration across the interalveolar septum. Am Rev Respir Dis 147: 168, 1993

Dri P, Cramer R, Romano M, Spessotto P, PatriarcaP: Effect of biological surfaces on neutrophil O2- production and its relationship to the CD11/CD18 integrin-dependent adherence. Int J Tissue React: 13, 193, 1991

Drost EM, Selby C, Lannan S, Lowe GO, MacNee W: Changes in neutrophil deformability following in vitro smoke exposure. Am J Resp Cell Mol Biol 6: 287, 1992

Duan H, Dixit VM: RAIDD is a new 'death' adaptor molecule. Nature 385: 86, 1997

Dubravek DB, Spriggs DR, Mannick JA, Rodrick ML: Circulating human peripheral granulocytes synthesize and secrete tumour necrosis factor α. Proc Natl Acad Sci USA 87: 6758, 1990

Dudley DT, Pang L, Decker SJ, Bridges AJ, Salteil AR: A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci USA 92: 7686, 1995

Duke RC, Chervenak R, Cohen JJ: Endogenous endonuclease-induced DNA fragmentation: An early event in cell-mediated cytolysis. Proc Natl Acad Sci USA 80: 6361, 1983

Duke RC, Cohen JJ: IL-2 addiction: withdrawal of growth factor activates a suicide program in dependent T cells. Lymphokine Res 5: 289, 1986

Durkop H, Latza U, Hummel M, Eitelbach F, Seed B, Stein H: Molecular cloning and expression of a new member of the nerve growth factor receptor that is characteristic for Hodgkin's disease. Cell 68: 421, 1992

Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA: Induction by IL1 and interferon-γ: tissue distribution, biochemistry and function of a natural adherence molecule (ICAM-1). J Immunol 137: 245, 1986

Duvall E, Wyllie AH: Death and the cell. Immunol Today 7: 115, 1986

Eck MJ, Beutler B, Kuo G, Merryweather JP, Sprang SR: Crystallization of trimeric recombinant human tumour necrosis factor (cachectin). J Biol Chem 263: 12 816, 1988

Eck MJ, Ultsch M, Rinderknecht E, de Vos AM, Sprang SR: The structure of human lymphotoxin (tumour necrosis factor-beta) at 1.9-A resolution. J Biol Chem 267: 2119, 1992

Eggleton P, Penhallow J, Crawford N: Priming action of inositol hexakisphosphate ($InsP_6$) on the stimulated respiratory burst in human neutrophils. Biochim Biophys Acta 1094: 309, 1991

Engelmann H, Aderka D, Rubinstein M, Rotman D, Wallach D: A tumour necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumour necrosis factor toxicity. J Biol Chem 264: 11974, 1989

Erickson SL, de Sauvage FJ, Kikly K, Carver-Moore K, Pitts-Meek, S, Gillett N, Sheehan KC, Schreiber RD, Goeddel DV, Moore MW: Decreased sensitivity to tumour necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature 372: 560, 1994

Espevik T, Nissen-Meyer J: A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumour necrosis factor from human monocytes. J Immunol Methods 95: 99, 1986

Espevik T, Brockhaus M, Loetscher H, Nonstad U, Shalaby R: Characterization of binding and biological effects of monoclonal antibodies against a human tumour necrosis factor receptor. J Exp Med 171: 415, 1990

Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM: Exposure of phosphatidyserine on the surface of apoptotic lymphocytes triggers specific recognition and recognition and removal by macrophages. J Immunol 148: 2207, 1992

Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G, Alnemri ES: In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. Proc Natl Acad Sci USA 93: 7464, 1996

Fiers W: The natural immune system: humoral factors, in Sim E (ed), IRL Press, Oxford, 1993, p65

Fletcher MP, Gallin JI: Human neutrophils contain an intracellular pool of putative receptors for the chemoattractant N-formyl-methionyl-leucyl-phenylalanine. Blood 62: 792, 1983

Freshney NW, Rawlinson L, Guesdon F, Jones E, Crowley S, Hsuan J, Saklatvala J: Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. Cell 78: 1039, 1994

Gamble JR, Harlan JM, Klebanoff SJ, Vadas MA: Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc Natl Acad Sci USA 82: 8667, 1985

Gatanaga T, Hwang C, Kohr W, Cappuccini F, Lucci III JA, Jeffes EWB, Lentz R, Tomich J, Yamamoto RS: Purification and characterization of an inhibitor (soluble tumor necrosis factor receptor) for tumour necrosis factor and lymphotoxin obtained from the serum ultrafiltrates of human cancer patients. Proc Natl Acad Sci USA 87: 8781, 1990

Genestier L, Bonnefoy-Berard N, Rouault J-P, Flacher M, Revillard J-P: Tumour necrosis factor-α up-regulates Bcl-2 expression and decreases calcium-dependent apoptosis in human B cell lines. Int Immunol 7: 533, 1995

Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenbeele P, Delvaux A, Fiers W, Goldman M, Velu T: Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. J Exp Med 177: 547, 1993

Glucksmann A: Cell deaths in normal vertebrate ontogeny. Biol Revs 26: 59, 1951

Goodwin RG, Anderson D, Jerzy R, Davis T, Brannan CI, Copeland NG, Jenkins NA, Smith CA: Molecular cloning and expression of the type I and type II murine receptors for tumour necrosis factor. Mol Cell Biol 11: 3020, 1991

Goodwin RG, Alderson MR, Smith CA, Armitage RJ, VandenBos T, Jerzy R, Tough TW, Schoenborn MA, Davis-Smith T, Hennen K, : Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. Cell 73: 447, 1993a

Goodwin RG, Din WS, Davis-Smith T, Anderson DM, Gimpel SD, Sato TA, Maliszewski CR, Brannan CI, Copeland NG, Jenkins NA et al.,: Molecular cloning of a ligand for the inducible T cell gene 4-1BB: a member of an emerging family of cytokines with homology to tumor necrosis factor. Eur J Immunol 23: 2631, 1993b

Gottlieb RA, Giesing HA, Zhu JY, Engler RL, Babior BM: Cell acidification in apoptosis: Granulocyte colony-stimulating factor delays programmed death in

neutrophils by up-regulating the vacuolar H^+-ATPase. Proc Natl Acad Sci USA 92: 5965, 1995

Granger GA, Williams TW: Lymphocyte toxicity in vitro: Activation and release of a cytotoxic factor. Nature 218: 1253, 1968

Gray PW, Aggarwal BB, Benton CV, Bringman TS, Henzel WJ, Jarrett JA, Leung DW, Moffat B, Ng P, Svedersky LPS, Palladino MA, Nedwin GE: Cloning and expression of cDNA for human lymphotoxin, a lymphokine with tumour necrosis activity. Nature 312: 721, 1984

Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopoulos S, Lesslauer W, Kollias G, Pfizenmaier K, Scheurich P: The transmembrane form of tumour necrosis factor is the prime activating ligand of the 80 kDa tumour necrosis factor receptor. Cell 83: 793, 1995

Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, Tedder T: Granulocyte-macrophage colony stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes and their precursors. J Immunol 145: 576, 1990

Grigg JM, Savill JS, Sarraf C, Haslett C, Silverman M: Neutrophil apoptosis and clearance from neonatal lungs. Lancet 338: 720, 1991

Gruss HJ, Boiani N, Williams DE, Armitage RJ, Smith CA, Goodwin RG: Pleotropic effects of the CD30 ligand on CD30-expressing cells and lymphoma cell lines. Blood 83: 2045, 1994

Guthrie LA, McPhail LC, Henson PM, Johnston RB Jnr: Priming of neutrophils for enhanced release of oxygen metabolites: evidence for increased activity of the superoxide-producing enzyme. J Exp Med 160: 1656, 1984

Hachiya O, Takeda Y, Miyata H, Wantanabe H, Yamashita T, Sendo F: Inhibition by bacterial lipopolysaccharide of spontaneous and TNFα-induced human neutrophil apoptosis in vitro. Microbiol Immunol 39: 715, 1995

Hahn T, Toker L, Budilovsky S, Aderka D, Eshhar Z, Wallach D: Use of monoclonal antibodies to a human cytotoxin for its isolation and for examining the self-induction of resistance to this protein. Proc Natl Acad Sci USA 82: 3814, 1985

Hall SE, Savill JS, Henson PM, Haslett C: Apoptotic neutrophils are phagocytosed by fibroblasts with participation of the fibroblast vitronectin receptor and involvement of a mannose/fructose-specific lectin. J Immunol 153: 3218, 1994

Han J, Lee JD, Bibbs L, Ulevitch RJ: A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265: 808, 1994

Hannah S, Mecklenburgh K, Rahman I, Bellingan GJ, Greening A, Haslett C, Chilvers ER: Hypoxia prolongs neutrophil survival in vitro. FEBS Lett 372: 233, 1995

Hannun YA, Loomis CR, Merrill AH Jr, Bell RM: Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets J Biol Chem 261: 12604, 1986

Hartwell JL, Shear MJ, Adams JRJ: Chemical treatment of tumours. VII. Nature of the hemorrhage-producing fraction from *Serratia marcescens (Bacillus prodigiosus)* culture filtrate. J Natl Cancer Inst 4: 107, 1943

Haslett C, Guthrie LA, Kopaniak MM, Johnstone RB, Henson PM: Modulation of multiple neutrophil functions by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am J Pathol 119: 101, 1985

Haslett C, Henson PM: Resolution of inflammation. In 'The Molecular and Cellular Biology of Wound Repair' Eds RAF Clark and PM Henson, Plenum Publishing Corp, New York. Pp185-211 1988

Haslett C, Savill JS, Meagher L: The neutrophil. Curr Opin Immunol 2: 10, 1989

Haslett C, Savill JS, Whyte MKB, Stern M, Dransfield I, Meagher LC: Granulocyte apoptosis and the control of inflammation. Phil Trans R Soc London B 345: 327, 1994

Hauser GJ, McIntosh JK, Travis WD, Rosenberg SA: Manipulation of oxygen radical-scavenging capacity in mice alters host sensitivity to tumor necrosis factor toxicity but does not interfere with its antitumor efficacy. Cancer Res 50: 3503, 1990

Hemmings BA: Akt signaling: linking membrane events to life and death decisions. Science 275: 628, 1997

Henson PM, Johnson RB Jr: Tissue injury in inflammation. Oxidants, proteinases and cationic proteins. J Clin Invest 79: 669, 1987

Hess S, Engelmann H: A novel function of CD40: induction of cell death in transformed cells. J Exp Med 183: 159, 1996

Higuchi M, Aggarwal BB: TNF induces internalization of the p60 receptor and shedding of the p80 receptor. J Immunol 152: 3550, 1994

Higuchi M, Singh S, Jaffrezou JP, Aggarwal BB: Acidic sphingomyelinase-generated ceramide is needed but not sufficient for TNF-induced apoptosis and nuclear factor-κB activation. J Immunol 156: 297, 1996

Hirose K, Longo DL, Oppenheim JJ, Matsushima K: Overexpression of mitochondrial manganese superoxide promets the survival of tumor cells exposed to interleukin-1, TNFa, selected anticancer drugs and ionizing radiation. FASEB J 7: 361, 1993

Hohmann HP, Remy R, Brockhaus M, van Loon AP: Two different cell types have different major receptors for human tumor necrosis factor (TNFα). J Biol Chem 264: 14927, 1989

Hohmann HP, Brockhaus M, Baeuerle PA, Remy R, Kolebeck R, van Loon AP: Expression of the types A and B tumor necrosis factor (TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NFκB. TNFα is not needed for induction of a biological effect via TNF receptors. J Biol Chem 265: 22409, 1990

Holtmann H, Wallach D: Down regulation of the receptors for tumour necrosis factor by interleukin 1 and 4β-phorbol-12-myristate-13-acetate. J Immunol 139: 1161, 1987

Howard M, muchamuel T, Andrade S, Menon S: Interleukin 10 protects mice from lethal endotoxemia. J Exp Med 177: 1205, 1993

Howard T, Li Y, Torres M, Guerrero A, Coates T: The 47-kD protein increased in neutrophil actin dysfunction with 47- and 89-kD protein abnormalities is lymphocyte-specific protein. Blood 83, 231, 1994

Hsu H, Xiong J, Goeddel DV: The TNF receptor 1-associated protein TRADD signals cell death and NFκB activation. Cell 81: 495, 1995

Hsu H, Shu H-B, Pan M-G, Goeddel DV: TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell 84: 299, 1996a

Hsu H, Huang J, Shu H-B, Baichwal V, Goeddel DV: TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signalling complex. Immunity 4: 387, 1996b

Hunter T: Protein kinases and phosphatases: The ying and yang of protein phosphorylation and signalling. Cell 80: 225, 1995

Hurley JV: Termination of inflammation, in Hurley JV (ed): Resolution in acute Inflammation, vol I. London, Churchill Livingstone, 1983, p. 169.

Ichinose Y, Hara N, Ohtu M, Aso H, Chikama H, Kawasaki M, Kubota I, Shimizu T, Yagawa K: Recombinant granulocyte colony-stimulating factor and

lipopolysaccharide maintain the phenotype of and superoxide anion generation by neutrophils. Infect Immun 58: 1647, 1990

Ishihara H, Martin BL, Brautigan DL, Karaki H, Ozaki H, Kato Y, Watabe N, Hashimoto K, Uemura K: Calyculin A and okadaic acid: Inhibitors of protein phosphatase activity. Biochem Biophys Res Commun 159: 871, 1989

Ishii Y, Lo SK, Malik AB: Neutrophil adhesion to TNFα-activated endothelial cells potentiates leukotriene B_4 production. J Cell Physiol 153: 187, 1992

Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66: 233, 1991

Itoh N, Nagata S: A novel protein domain required for apoptosis: mutational analysis of human FAS antigen. J Biol Chem 268: 10932, 1993

Jacob CO, Fronek Z, Lewis GD, Koo M, Hansen JA, McDervitt HO: Heritable major histocompatibility complex II-associated differences in production of tumor necrosis factor α: relevance to genetic predisposition to systemic lupus erythematosus. Proc Natl Acad Sci USA 87: 1233, 1990

Jaffrezou JP, Herbert JM, Levade T, Gau MN, Chatelain P, Laurent G: reversal of mulyidrug resistance by calcium channel blocker SR33557 without photoaffinity labeling of P-glycoprotein. J Biol Chem 266: 19858, 1991

Jaffrezou JP, Levade T, Chatelain P, Laurent G: Modulation of subcellular distribution of doxorubicin in multidrug-resistant P388/ADR mouse leukemia cells by the chemosensitizer ((2-isopropyl-1-(4-[3-*N*-methyl-*N*-(3,4-dimethoxy-beta-phenethyl)amino]propyloxy)-benzenesulphonyl)) indolizine. Cancer Res 52: 6440, 1992

Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA, Grant S: Induction of apoptotic DNA damage and cell death by activation of the sphingomyelinase pathway. Proc Nat Acad Sci USA 91: 73, 1994

Jenkinson EJ, Kingston R, Smith CA, Williams GT, Owen JJT: Antigen-induced apoptosis in developing T cells: a mechanism for negative selection of the T cell receptor repertoire. Eur J Immunol 19: 2175, 1989

Jesaitis AJ, Naemura JR, Painter RG, Sklar LA, Cochrane CG: Intracellular localization of *N*-formyl chemotactic receptor and Mg^{2+}-dependent ATPase in human granulocytes. Biochim Biophys Acta 719: 556, 1982

Johnson, D, Lanahan A, Buck CR, Sehgal A, Morgan C, Mercer E, Bothwell M, Chao M: Expression and structure of the human NGF receptor. Cell 47: 545, 1986

Jones AL, Selby P: Tumour necrosis factor: clinical relevance. Cancer Surveys 8: 817, 1989

Jutila MA, Rott, L, Berg EL, Butcher EC: Function and regulation of the neutrophil Mel-14 antigen in vivo: comparison with LFA-1 and Mac-1. J Immunol 143: 3318, 1989

Kahler H, Shear MJ, Hartwell JL: Chemical treatment of tumours. VIII. Ultracentrifugal and electrophoretic analysis of the hemorrhage-producing fraction from *Serratia marcescens (Bacillus prodigiosus)* culture filtrate. J Natl Cancer Inst 3: 123, 1943

Karin M: The regulation of AP-1 activity by mitogen-activated protein kinases. J Biol Chem 270: 16483, 1995

Kato T, Takeda Y, Nakada T, Sendo F: Inhibition by dexamethasone of human neutrophil apoptosis in vitro. Nat Immun 14: 198, 1995

Kawakami M, Cerami A: Studies of endotoxin-induced decrease in lipoprotein lipase activity. J Exp Med 154: 631, 1981

Kawakami M, Pekala PH, Lane DM, Cerami A: Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells. Proc Natl Acad Sci USA 79: 912, 1982

Kerr JFR, Wyllie AH, Currie AR: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26: 239, 1972

Kilbourn RG, Gross SS, Jubran A, Adams J, Griffith OW, Levi R, Lodato RF: NG-methyl-L-arginine inhibits tumor necrosis factor-induced hypotension: implications for the involvement of nitric oxide. Proc Natl Acad Sci USA 87: 3629, 1990

Kim YM, De Vera ME, Watkins SC, Billiar TR: Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein expression. J Biol Chem 272: 1402, 1997

Kinkhabwala M, Sehajpal P, Skolnik E, Smith D, Sharma VK, Vlassara H, Cerami A, Suthanthiran M: A novel addition to the T cell repertory. Cell surface expression of tumour necrosis factor/cachectin by activated normal human T cells. J Exp Med 171: 941, 1990

Kishimoto TK, Jutila AM, Berg EL, Butcher EC: Neutrophil Mac-1 and Mel-14 adhesion proteins inversely regulated by chemotactic factors. Science 245: 1238, 1989

Kitchen E, Condliffe AM, Rossi AG, Haslett C, Chilvers ER: Characterization of inositol hexakisphosphate ($InsP_6$)-mediated priming in human neutrophils: lack of extracellular [^{3}H]-$InsP_6$ receptors. Br J Pharmacol 117: 979, 1996a

Kitchen E, Rossi AG, Condliffe AM, Haslett C, Chilvers ER: Demonstration of reversible priming of human neutrophils using platelet-activating factor. Blood 88: 4330, 1996b

Kitchen E: Ph D Thesis, University of Edinburgh, 1997

Kitson J, Raven T, Jiang Y-P, Goeddel DV, Giles KM, Pun K-Y, Grinham CJ, Brown R, Farrow SN: A death-domain-containing receptor that mediates apoptosis. Nature 384: 372, 1996

Klebanoff SJ, Vadas MA, Harlan JM, Sparks LH, Gamble JR, Agosti JM, Waltersdorph AM: Stimulation of neutrophils by tumor necrosis factor. J Immunol 136: 4220, 1986

Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD: The release of cytocrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132, 1997

Kolesnick R, Golde DW: The sphingomyelin pathway in tumor necrosis factor and interleukin-1 signalling. Cell 77: 325, 1994

Kreigler M, Perez C, DeFay K, Albert I, Lu SD: A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications of the complex physiology of TNF. Cell 53: 45, 1988

Kroemer G: Mitochondrial control of nuclear apoptosis. J Exp Med 183: 1533, 1996a

Kubes P: Nitric oxide affects microvascular permeability in the intact and inflamed microvasculature. Microcirculation 2: 235, 1995

Kunkel SL, Lukacs N, Strieter RM: Expression and biology of neutrophil and endothelial cell-derived chemokines. Semin Cell Biol 6: 327, 1995

Kwon MS, Heinz H, Abid H, Leibold W: Differential influences of recombinant TNFα and recombinant IFN-γ on purified polymorpholeukocytes in vitro, in Bonavida, Gifford, Kircher, Old (eds): Tumour Necrosis Factor/Cachectin and Related Cytokines. Basal, Karger, 1988, p 87

Kwon BS, Weissman SM: cDNA sequences of two inducible T-cell genes. Proc Natl Acad Sci USA 86: 1963, 1989

LaMarre J, Wollenberg GK, Gonias SL, Hayes MA: Cytokine binding and clearance properties: activated α_2-macroglobulins. Lab Invest 65: 3, 1991

Lad PM, Goldberg BJ, Smiley PA, Olson CV: receptor-specific threshold effects of cAMP are involved in the regulation of enzyme release and superoxide production from human neutrophils. Biochim Biophys Acta 846: 286, 1985

Lange-Carter CA, Pleiman CM, Gardner AM, Blumer KJ, Johnson GL: A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 260: 315, 1993

Lantz M, Bjornberg F, Olsson I, Richter J: Adherence of neutrophils induces release of soluble tumor necrosis factor receptor forms. J Immunol 152: 1362, 1994

Larrick JW, Graham D, Toy K, Lin LS, Senyk G, Fendly BM: Recombinant tumor necrosis factor causes activation of human granulocytes. Blood 69: 640, 1987

Laudanna C, Rossi F, Berton, G: Effect of inhibitors of distinct signalling pathways on neutrophil O_2^- generation in response to tumour necrosis factor-α and antibodies against CD18 and CD11a: Evidence for a common and unique pattern of sensitivity to wortmannin and protein tyrosine kinase inhibitors. Biochem Biophys Res Commun 190: 935, 1993

Lavoie JN, Lambert H, Hickey E, Weber LA, Landry J: Modulation of cellular thermoresistance and actin filament stability accompanies phosphorylation-induced changes in the oligomeric structure of heat shock protein 27. Mol Cell Biol 15: 505, 1995

Lee A, Whyte MKB, Haslett C: Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol 54: 283, 1993

Lee BSY, Park CG, Choi Y: T cell receptor-dependent cell death of T cell hybridomas mediated by the CD30 cytoplasmic domain in association with tumor necrosis factor receptor-associated factors. J Exp Med 183: 669, 1996

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, Strickler JE, McLaughlin MM, Siemens IR, Fisher S, Livi GP, White JR, Adams JL, Young PR: A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 373: 739, 1994

Lee SY, Lee SY, Choi Y: TRAF-interacting protein (TRIP): A novel component of the tumor necrosis factor receptor (TNFR)- and CD30-TRAF signaling complex that inhibits TRAF2-mediated NF-κB activation. J Exp Med 185: 1275, 1997

Lennon SV, Martin SJ, Cotter TG: Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Prolif 24: 203, 1991

Lewis M, Tartaglia LA, Lee A, Bennett GL, Rice GC, Wong GHW, Chen EY, Goeddel DV: Cloning and expression of cDNAs for two distinct murine tumour necrosis factor receptors demonstrate one receptor is species specific. Proc Natl Acad Sci USA 88: 2830, 1991

Liles WC, Kiener PA, Ledbetter JA, Aruffo A, Klebanoff SJ: Differential expression of Fas (CD95) and Fas ligand on human neutrophils: Implications for the regulation of apoptosis in neutrophils. J Exp Med 184: 429, 1996

Lin A, Minden A, Martinetto H, Claret F-X, Lange-Carter C, Mercurio F, Johnson GL, Karin M: Identification of a dual-specificity kinase that activates the Jun kinases and p38-Mpk2. Science 268: 286, 1995

Lindemann A, Riedel D, Oster W, Ziegler-Heitbrock HWL, Mertelsmann R, Hermann F: Granulocyte-macrophage colony-stimulating factor induces cytokine production by human polymorphonuclear leukocytes. J Clin Invest 83: 1308, 1989

Liu ZG, Hsu H, Goeddel DV, Karin M: Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell 87: 565, 1996

Lo SK, Everitt J, Gu J, Malik AB: Tumour necrosis factor mediates experimental pulmonary edema by ICAM-1 and CD18-dependent mechanisms. J Clin Invest 89: 981, 1992

Loetscher H, Pan YC, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W: Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. Cell 61: 351, 1990

Loetscher H, Gentz R, Zulauf M, Lustig A, Tabuchi H, Schlaeger EJ, Brockhaus M, Gallati H, Manneberg M, Lesslauer W: Recombinant 55-kD tumour necrosis factor (TNF) receptor. Stoichiometry of binding to TNF alpha and TNF beta and inhibition of TNF activity. J Biol Chem 266: 324, 1991

Loetscher H, Stueber D, Banner D, Mackay F, Lesslauer W: Human tumor necrosis factor α (TNFα) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors. J Biol Chem 268: 26350, 1993

Lomo J, Blomhoff HK, Beiske K, Stokke T, Smeland EB: TGF-beta 1 and cyclic AMP promote apoptosis in resting human B lymphocytes. J Immunol 154: 1634, 1995

Lopez AF, Williamson DJ, Gamble JR, Begley CG, Harlan JM, Klebanoff SJ, Waltersdorf A, Wong G, Clark SC, Vadas MA: Recombinant human granulocyte-macrophage colony-stimulating factor stimulates in vitro mature human neutrophil and eosinophil function, surface receptor, and survival. J Clin Invest 78: 1220, 1986

Loscalzo J, Welch G: Nitric oxide and its role in the cardiovascular system. Prog Cardiovasc Dis 38: 87, 1995

Mackay F, Loetscher H, Stueber D, Gehr G, Lesslauer W: Tumor necrosis factor alpha (TNF-α)-induced cell adhesion to human endothelial cells is under dominant control of one TNF receptor subtype, TNF-R55. J Exp Med 177: 1277, 1993

MacNee W, Selby C: Neutrophil kinetics in the lungs. Clin Sci 79: 97, 1990

Malinin NL, Boldin MP, Kovalenko AV, Wallach D: MAP3K-related kinase involved in NFκB induction by TNF, CD95 and IL-1. Nature 385: 540, 1997

Mallett S, Barclay AN: A new superfamily of cell surface proteins related to the nerve growth factor receptor. Immunol Today 12: 220, 1991

Mangan DF, Wahl SM: Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J Immunol 147: 3408, 1991

Mannel DN, Moore RN, Mergenhagen SE: Macrophages as a source of tumouricidal activity (tumour-necrotizing factor). Infect Immun 30: 523, 1980

Mannion BA, Weiss J, Elsbach P: Separation of sublethal and lethal effects of polymorphonuclear leukocytes on *Escherichia coli*. J Clin Invest 86: 631, 1990

Marshall CJ: specificity of receptor tyrosine kinase signalling: Transient versus sustained extracellular signal-related kinase activation. Cell 80: 179, 1995

Marsters SA, Sheridan JP, Donahue CJ, Pitti RM, Gray CL, Goddard AD, Bauer KD, Ashkenazi A: Apo-3, a new member of the tumor necrosis factor receptor family, contains a death domain and activates apoptosis and NFκB. Current Biol 12: 1669, 1996

Matsuda M, Masutani H, Nakamura H: Protective activity of adult T cell leukemia-derived factor (A DF) against human TNFα-dependent cytotoxicity on U937 cells. J Immunol 147: 3837, 1991

Matthew N, Neale ML, Jackson SK, Stakk JM: Tumour killing by TNFα, inhibition by anaerobic conditions, free radical scavengers and inhibitors of arachidonate metabolism. Immunol 62: 153, 1987

McConkey DJ, Orrenius S, Jondal M: Agents that elevate cAMP stimulate DNA fragmentation in thymocytes. J Immunol 145: 1227, 1990

McGregor PE, Agrawal DK, Edwards JD: Attenuation of human leukocyte adherence to endothelial cell monolayers by tyrosine kinase inhibitors. Biochem Biophys Res Comm 198: 359, 1994

McGuire W, Hill AVS, Allsopp CEM, Greenwood BM, Kwjatkowski D: Variation in the TNF-α promoter region associated with susceptibility to cerebral malaria. Nature 371: 508, 1994

Meagher LC, Savill JS, Baker A, Fuller R, Haslett C: Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B_2. J Leukoc Biol 52: 269, 1992

Meagher LC, Cousin JM, Seckl JR, Haslett C: Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol 156: 4422, 1996

Menegazzi R, Cramer R, Patriarca P, Scheurich P, Dri P: Evidence that tumor necrosis factor-α (TNF)-induced activation of neutrophil respiratory burst on biologic surfaces is mediated by the p55 TNF receptor. Blood 84: 287, 1994

Merril AH Jr, Sereni AM, Stevens VL, Hannun YA, Bell RM, Kinkade JM Jr: J.Biol Chem 261: 12610, 1986

Metchnikoff E: Lectures on the Comparative Pathology of Inflammation, Lecture VII (F.A. Starling and E.H. Starling, translators), Dover Publications, New York, 1891, p 106.

Minden A, Lin A, McMahon M, Lange-Carter C, Derijard B, Davis RJ, Johnson GL, Karin M: Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 266: 1719, 1994

Minshal C, Arkins S, Freund GG: Requirement for phosphatidylinositol 3'-kinase to protect hemopoietic progenitors against apoptosis depends upon the extracellular survival factor. J Immunol 156: 939, 1996

Miyamoto S, Verma IM: Rel/NF-κB/IκB story. Adv Cancer Res 66: 255, 1995

Mohler KM, Sleath PR, Fitzner JN, Cerretti DP, Alderson M, Kerwar SS, Torrance DS, Otten-Evans C, Greenstreet T, Weerawarna K, Kronheim SR, Petersen M, Gerhart M, Kozolosky CJ, March CJ, Black RA: Protection against a lethal dose of endotoxin by an inhibitor of tumour necrosis factor processing. Nature 370: 218, 1994

Moilanen E, Vapaatalo H: Nitric oxide in inflammation and immune responses. Ann Med 27: 359, 1995

Molvig J, Baek L, Christensen P et al.,: Endotoxin-stimulated human monocyte secretion of interleukin-1, tumor necrosis factor alpha, and prostaglandin E_2 shows stable interindividual differences Scand J Immunol 27: 705, 1988

Monastra G, Cabrelle A, Zambon A, Rosato A, Macino B, Collavo D, Zanovello P: Membrane form of TNFα induces both cell lysis and apoptosis in susceptible target cells. Cell Immunol 171: 102, 1996

Morris RG, Hargreaves AD, Duvall E, Wyllie AH: Hormone induced cell death 2. Surface changes in thymocytes undergoing apoptosis. Am J Path 115: 426, 1984

Mosialos G, Birkenbach M, Yalamanchili R, VanArsdale T, Ware C, Kieff E: The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell 80: 389, 1995

Mosselmans R, Hepburn A, Dumont JE, Fiers W, Galand P: Endocytic pathway of recombinant murine tumour necrosis factor in L929 cells. J Immunol 141: 3096, 1988

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell 85: 817, 1996

Nagata S, Goldstein P: The Fas death factor. Science 267: 1449, 1995

Nahas N, Molski TFP, Fernandez GA, Sha'afi RI: Tyrosine phosphorylation and activation of a new mitogen-activated protein (MAP)-kinase cascade in human neutrophils stimulated with various agonists. Biochem J 318: 247, 1996

Nathan CF: Neutrophil activation on biological surfaces. Massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. J Clin Invest 80: 1550, 1987

Nathan C, Srimal S, Farber C, Sanchez E, Kabbasch L, Asch A, Gailit J, Wright SD: Cytokine-induced respiratory burst of human neutrophils: Dependence on extracellular matrix proteins and CD11/CD18 integrins. J Cell Biol 109: 1341, 1989

Nathan C, Sanchez E: Tumor necrosis factor and CD11/CD18 (β_2) integrins act synergistically to lower cAMP in human neutrophils. J Cell Biol 111: 2171, 1990

Nedwin GE, Naylor SL, Sakaguchi AY, Smith D, Jarrett-Nedwin J, Pennica D, Goeddel DV, Gray PW: Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. Nucleic Acids Res 13: 6361, 1985

Newman SL, Henson JE, Henson PM: Phagocytosis of senescent neutrophils by human monocyte-derived macrophages. J Exp Med 156: 430, 1982

Nick JA, Avdi AN, Gerwins P, Johnson GL, Worthen GS: Activation of a p38 mitogen-activated protein kinase in human neutrophils by lipopolysaccharide. J Immunol 156: 4867, 1996

Nicoletti I, Migliorati G, Oagliacci MC, Grignani F, Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 139: 271, 1991

Niwa M, Yousif A-EL, Kohno K-I, Kanamori Y, Matsuno M, Abe A, Uematsu T: The loss of recombinant human granulocyte colony-stimulating factor and recombinant human TNF-a priming effects on the superoxide-generating response in exudated neutrophils is associated with a decrease in their receptor affinities. J Immunol 157: 4147, 1996

Novogrodsky A, Vanichkin A, Patya M, Gazit A, Osherov N, Levitzki: Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase activity. Science 264: 1319, 1994

Nunn AVW, Barnard ML, Bhakoo K, Murray J, Chilvers ER, Bell JD: Characterisation of secondary metabolites associated with neutrophil apoptosis. FEBS Lett 392: 295, 1996

O'Malley WE, Achinstein B, Shear MJ: Action of bacterial polysaccharide on tumours. II. Damage of sarcoma 37 by serum of mice treated with Serratia marcescens polysaccharide, and induced tolerance. J Natl Cancer Inst 29: 1169, 1962

Obeid LM, Linardic CM, Karolak LA, Hannun YA: Programmed cell death induced by ceramide. Science 259: 1769, 1993

Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhein J, Trauth BC: Purification and molecular cloning of the APO-1 surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. J Biol Chem 267: 10709, 1992

Ohta H, Yatomi Y, Sweeney EA, Hakomori S, Igarashi Y: A possible role of sphingosine in induction of apoptosis by tumour necrosis-α in human neutrophils. FEBS lett 355: 267, 1994

Olsson I, Lantz M, Nilsson E, Peetre C, Thysell H, Grubb A, Adolf G: Isolation and characterization of a tumor necrosis factor binding protein from urine. Eur J Haematol 42: 270, 1989

Opdenakker G, Van Damme J: Cytokines and proteases in invasive processes: molecular similarities between inflammation and cancer. Cytokine 4: 251, 1992

Osborn L, Kunkel S, Nabel GJ: Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc Natl Acad Sci USA 86: 2336, 1986

Paul A, Cuenda A, Bryant CE, Murray J, Chilvers ER, Cohen P, Gould GW, Plevin R: Involvement of MAP kinase homologues in lipopolysaccharide-mediated induction of cyclo-oxygenase-2 and the inhibition of cellular apoptosis. Submitted to Br J Pharmacol

Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB, Goeddel DV: Human tumour necrosis factor, precursor structure, expression and homology to lymphotoxin. Nature 312: 724, 1984

Perez C, Albert I, DeFay K, Zachariades N, Gooding L, Kreigler M: A nonsecrestable cell surface mutant of tumour necrosis factor (TNF) kills by cell-to-cell contact. Cell 63: 251, 1990

Petersen M, Steadman R, Hallett MB, Matthews N, Williams JD: Zymosan-induced leukotriene B_4 generation by human neutrophils is augmented by rhTNF-α but not chemotactic peptide. Immunology 70: 75, 1990

Peetre C, Thysell H, Grubb A, Olsson I: A tumour necrosis factor binding protein is present in human biological fluids. Eur J Haematol 41: 414, 1988

Pfeffer K, Matsuyama T, Kündig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW: Mice deficient for the 55 kd tumour necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell 73: 457, 1993

Picot F, Briant L, Jongeneel CV et al.,: Association of tumor necrosis factor (TNF) and class II major histocompatibility complex alleles with the secretion of TNFα and TNFβ by human mononuclear cells: a possible link to insulin-dependent diabetes mellitus. Eur J Immunol 23: 224, 1993

Pons F, Williams TJ, Kirk SA, McDonald F, Rossi AG: Pro-inflammatory and anti-inflammatory effects of the stable prostaglandin D2 analogue ZK 118.182. Eur J Pharmacol 261: 237, 1994

Porteu F, Nathan C: Shedding of tumour necrosis factor receptor by activated human neutrophils. J Exp Med 172: 1990, 1990

Porteu F, Hieblot C: Tumor necrosis factor induces a selective shedding of its p75 receptor from human neutrophils. J Biol Chem 269: 2834, 1994

Price TH, Dale DC: Neutrophil preservation: the effect of short-term storage on in vivo kinetics. J Clin Invest 59: 475, 1977

Probert L, Akassoglou K, Alexopoulou L, Douni E, Haralambous S, Hill S, Kassiotis G, Kontoyiannis D, Pasparakis M, Plows D, Kollias G: Dissection of the pathologies induced by transmembrane and wild-type tumor necrosis factor in transgenic mice. J Leukoc Biol 59: 518, 1996

Pushkareva M, Obeid LM, Hannun YA: Ceramide: an endogenous regulator of apoptosis and growth suppression. Immunol Today 16: 294, 1995

Qu J, Condliffe AM, Lawson M, Plevin RJ, Riemersma RA, Barclay GR, McClelland DB, Chilvers ER: Lack of effect of recombinant platelet-derived growth factor on human neutrophil function. J Immunol 154: 4133, 1995

Rabizadeh S, Bredesen DE: Is p75NGFR involved in developmental neural cell death? Dev Neurosci 16: 207, 1994

Rafinee P, Lee JK, Leung CC, Raffin TA: TNFα induces tyrosine phosphorylation of mitogen-activated protein kinase in adherent human neutrophils. J Imunol 154: 4785, 1995

Raingeaud J, Gupta S, Rogers JS, Dickens M, Han J, Ulevitch RJ, Davis RJ: Pro-inflammatory cytokine and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. J Biol Chem 270: 7420, 1995

Rakita, RM, Michel BR, Rosen H: Myeloperoxidase-mediated inhibition of microbial respiration: damage to *Escherichia coli* ubiquinol oxidase. Biochemistry 28: 3031, 1989

Ratcliffe DR, Nolin SL, Cramer EB: Neutrophil interaction with influenza-infected epithelial cells. Blood 72: 142, 1988

Rees DD, Palmer RMJ, Schulz R, Hodson HF, Moncada S: Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. Br J Pharmacol 101: 746, 1990

Remick DG, Strieter RM, Eskandari MK, Nguyen DT, Genord MA, Kunkel SL: Role of tumor necrosis factor-a in lipopolysaccharide-induced pathologic alterations. Am J Path 136: 49, 1990

Ren Y, Savill JS: Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J Immunol 154: 2366, 1995

Richter J, Andersson T, Olsson I: Effect of tumour necrosis factor and granulocyte/macrophage colony-stimulating factor on neutrophil degranulation. J Immunol 142: 3199, 1989

Richter J, Gullberg U, Lantz M: TNF-induced superoxide anion generation in human neutrophils involves both the p55 and p75 TNF receptor. J Immunol 154: 4142, 1995

Robertson OH, Uhley CG: Changes occurring in the macrophage system of the lungs in pneumococcus lobar pneumonia. J Clin Invest 15: 115, 1938

Robinson J, Watson F, Bucknall RC, Edwards SW: Activation of neutrophil reactive oxidant production by synovial fluid from patients with inflammatory joint disease:

soluble and insoluble immunoglobulin aggregates activate different pathways in primed and unprimed cells. Biochem J 286: 345, 1992

Rosen GM, Pou S, Ramos CL, Cohen MS, Britigan BE: Free radicals and phagocytic cells. FASEB 9: 200, 1995

Rossi AG, Cousin JM, Dransfield I, Lawson MF, Chilvers ER, Haslett C: Agents that elevate cAMP inhibit human neutrophil apoptosis. Biochem Biophys Res Commun 21: 892, 1995

Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H: Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated cytotoxicity but highly susceptible to infection by *Listeria monocytogenes*. Nature 364: 798, 1993

Rothe M, Wong SC, Henzel WJ, Goeddel DV: A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumour necrosis factor receptor. Cell 78: 681, 1994

Rothe M, Pan M-G, Henzel WJ, Ayres TM, Goeddel DV: The TNFR2-TRAF signaling complex contains two novel proteins related to baculovirus inhibitor or apoptosis proteins. Cell 83: 1243, 1995a

Rothe M, Sarma V, Dixit VM, Goeddel DV: TRAF-2-mediated activation of NFkB by TNF receptor 2 and CD40. Science 269: 1424, 1995b

Rouse J, Cohen P, Trigon S, Morange M, Alonso-Liamazares A, Zamanillo D, Hunt T, Nebreda AR: A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase 2 and phosphorylation of the small heat shock proteins. Cell 78: 1027, 1994

Rouzer CA, Cerami A: Hypertriglyceridemia associated with *Trypanosoma brucei brucei* infection in rabbits: Role of defective triglyceride removal. Mol Biochem Parasitol 2: 31, 1980

Ruddle NH, Waksman BT: Cytotoxic effect of lymphocyte-antigen interaction in delayed hypersensitivity. Science 157: 1060, 1967

Ruddle NH, Waksman BT: Cytotoxicity mediated by soluble antigens and leucocytes in delayed hypersensitivity I. Characterisation of the phenomenon. J Exp Med 128: 1237, 1968a

Ruddle NH, Waksman BT: Cytotoxicity mediated by soluble antigens and leucocytes in delayed hypersensitivity II. Correlation of the in vitro response with skin reactions. J Exp Med 128: 1255, 1968b

Russ MR, Gifford GE: tumour necrosis factor. In Lymphokines Vol 2 (ed E Pick), pp 235-72. Academic Press, New York, 1981

Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodget JR, Avruch J, Kyriakis JM, Zon LI: Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature 372:794, 1994

Savage COS, Rees AJ: Role of neutrophils in vasculitis. In: The Handbook of immunology: Immunopharmacology of neutrophils 259, Academic press, edited Hellewell PG and Williams TJ

Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to recognition by macrophages. J Clin Invest 83: 865, 1989a

Savill JS, Henson PM, Haslett C: Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge sensitive" recognition mechanism. J Clin Invest 84: 1518, 1989b

Savill JS, Dransfield I, Hogg N, Haslett C: Macrophage recognition of 'senescent self'; the vitronectin receptor mediates phagocytosis of cells undergoing apoptosis. Nature 342: 170, 1990

Savill JS, Smith J, Ren Y, Sarraf C, Abbott F, Rees AJ: Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. Kid Int 42: 924, 1992a

Savill JS, Hogg N, Haslett. C: Thrombospondin co-operates with CD36 and the vitronectin receptor in macrophage recognition of aged neutrophils. J Clin Invest 90: 1513, 1992b

Scannell G, Waxman K, Vaziri ND, Zhang J, Kaupke CJ, Jalali M, Hecht CC: Hypoxia-induced alterations of neutrophil membrane receptors. J Surg Res 59: 141, 1995

Schall TJ, Lewis M, Koller KJ, Lee A, Rice GC, Wong GHW, Gatalanga T, Granger GA, Lentz R, Raab H, Kohr WJ, Goeddel DV: Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell 61: 361, 1990

Schleiffenbaum B, Fehr J: The tumour necrosis factor receptor and human neutrophil function: deactivation and cross-deactivation of tumor necrosis factor-induced neutrophil responses by receptor down-regulation. J Clin Invest 86:184, 1990

Schleimer RP, Freeland HS, Peters SP, Brown KE, Derse CP: An assessment of the effects of glucocorticoids on degranulation, chemotaxis, binding to vascular endothelium and formation of leukotriene B_4. J Pharmacol Exp Ther 250: 598, 1989

Scleimer RP: Effects of glucocorticosteroids on inflammatory cells relevant to their therapeutic action. Am Rev Respir Dis 141: 559, 1990

Schmid-Schonbein GW, Usami S, Skalak R, Chien S: The interaction of leukocytes and erythrocytes in capillaries and post capillary venules. Microvasc Res 19: 45, 1980

Schuchmann M, Hess S, Bufler P, Brakebusch C, Wallach D, Porter A, Riethmuller G, Engelmann H: Functional discrepancies between tumour necrosis factor and lymphotoxin alpha explained by trimer stability and distinct receptor interactions. Eur J Immunol 25: 2183, 1995

Schulze-Ostoffe K, Bakker AC, Vanhaesebroeck B, Beyaert R, Jacob WA, Fiers: Cytotoxic activity of tumor necrosis factor is mediated by early damage of mitochondrial function. J Biol Chem 267: 5317, 1992

Seckinger P, Isaaz S, Dayer JM: Purification and biological characterization of a specific tumor necrosis factor alpha inhibitor. J Biol Chem 264: 11966, 1989

Sengelov H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N: Subcellular localization and dynamics of Mac-1 ($\alpha_m\beta_2$) in human neutrophils. J Clin Invest 93: 1467, 1993a

Sengelov H, Kjeldsen L, Borregaard N: Control of exocytosis in early neutrophil activation. J Immunol 150: 1535, 1993b

Shalaby MR, Aggarwal BB, Rinderknecht E, Svedersky LPS, Finkle BS, Palladino MA Jr: Activation of human polymorphonuclear neutrophil functions by interferon-γ and tumor necrosis factors. J Immunol 135: 2069, 1985

Shalaby MR, Palladino MA Jr, Hirabayashi SE, Eessalu TE, Lewis GD, Shepard HM, Aggarwal BB: Receptor binding and activation of polymorphonuclear neutrophils by tumor necrosis factor-alpha. J Leukoc Biol 41: 196, 1987

Shalaby MR, Sundan A, Loetscher H, Brockhaus M, Lesslauer W, Espevik T: Binding ans regulation of cellular functions by monoclonal antibodies against tumor necrosis factor receptors. J Exp Med 172: 1517, 1990

Shaw JO: Leukocytes in chemotactic fragment-induced lung inflammation: Vascular emigration and alveolar surface migration. Am J Path 101: 283, 1980

Shear MJ, Andervont HB: Chemical treatment of tumours. III. Separation of hemorrhage-producing fraction of *B. coli* filtrate. Proc Soc Exp Bio Med 34: 323, 1936

Shear MJ, Turner FC, Perrault A, Shovelton J: Chemical treatment of tumours. V. isolation of the hemorrhage-producing fraction from *Serratia marcescens (Bacillus prodigiosus)* culture filtrate. J Natl Cancer Inst 4: 81, 1943a

Shear MJ, Perrault A, Adams JRJ: Chemical treatment of tumours. VI. Method employed in determining the potency of hemorrhage-producing bacterial preparations. J Natl Cancer Inst 4: 99, 1943b

Shear MJ: Chemical treatment of tumours. Eactions of mice with primary subcutaneous tumours to injection of a hemorrhage-producing bacterial polysaccharide. J Natl Cancer Inst 4: 461, 1944

Simpson SQ, Singh R, Bice DE: Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-α production of murine macrophages. Am J Respir Cell Mol Biol 10: 284, 1994

Skerrett SJ, Bagby GJ, Nelson S: Antibody to tumour necrosis factor impairs pulmonary clearance of Legionella pneumophila. Am J Respir Crit Care Med 149: A871, 1994

Smith RA, Bagglioni C: The active form of tumour necrosis factor is a trimer. J Biol Chem 262: 6951, 1987

Smith CW, Martin SD, Rothlein R, Toman C, Anderson DC: Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils in vitro. J Clin Invest 83: 2008, 1989

Smith CA, Davis T, Anderson D, Solam L, Beckmann MP, Jerzy R, Dower SK, Cosman D, Goodwin RG: A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. Science 248: 1019, 1990

Smith RM, Curnutte JT: Molecular basis of chronic granulomatous disease. Blood 77: 673, 1991

Smith CA, Farrah T, Goodwin RG: The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76: 959, 1994

Solomskin J, Tindal CJ, Cave CM, Zemlan F: Regulation of TNF-alpha receptor expression in human neutrophils by various cell activating factors. J Surg Res 56: 261, 1994

Srinivasula SM, Ahmad M, Fernandes-Alnemri T, Litwack G, Alnemri ES: Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. Proc Natl Acad Sci USA 93: 14486, 1996

Stamenkovie I, Clark EA, Seed B: A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J 8: 1403, 1989

Stranger BZ, Leder P, Lee TH, Kim E, Seed B: RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. Cell 81: 513, 1995

Suda T, Takahashi T, Goldstein P, Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75: 1169, 1993

Susin SA, Zamzami N, Castedo M, Daugas E, Wang HG, Geley S, Fassay F, Reed JC, Kroemer G: The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. J Exp Med 186: 25, 1997

Swartz H, Tuckwell, J, Lotz, M: A receptor induced by lymphocyte activation (ILA): a new member of the human nerve-growth-factor/tumor necrosis-factor receptor family. Gene 134: 295, 1993

Tada H, Shiho O, Kuroshima K, Koyama M, Tsukamoto K: An improved colorometric assay for interleukin-2. J Immunol Methods 93: 157, 1986

Taguchi T, Sohmura Y: Clinical studies with TNF. Biotherapy 3: 177, 1991

Takeda Y, Watanabe H, Yonehara S, Yamashita T, Saito S, Sendo F: Rapid acceleration of neutrophil apoptosis by tumour necrosis factor-α. Int Immunol 5: 691, 1993

Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ: FGF and stress regulate CREB and AFT-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. EMBO J 15: 4629, 1996

Tartaglia LA, Ayres TM, Wong GHW, Goeddel DV: A novel domain within the 55kd TNF receptor signals cell death. Cell 74: 845, 1993a

Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: the 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa receptor. J Biol Chem 268: 18542, 1993b

Thompson HL, Matsushima K: Human polymorphonuclear leucocytes stimulated by tumour necrosis factor-alpha show increased adherence to extracellular matrix

proteins which is mediated via the CD11b/18 complex. Clin Exp Immunol 90: 280, 1992

Tiku K, Tiku ML, Skosey JL: Interleukin 1 production by human polymorphonuclear neutrophils. J Immunol 136: 3677, 1986

Toker A, Cantley L: Signalling through the lipid products of phosphoinositide-3-OH kinase. Nature 387: 673, 1997

Torti FM, Dieckmann B, Beutler B, Cerami, A, Ringold GM: A macrophage factor inhibits adipocyte gene expression: An in vitro model of cachexia. Science 229: 867, 1985

Tosi MF, and Zakem H: Surface expression of Fcγ receptor III (CD16) on chemoattractant-stimulated neutrophils is determined by both surface shedding and translocation from intracellular storage compartments. J Clin Invest 90: 462, 1992

Tracey KJ, Fong Y, Hesse DG et al.,: Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature 330: 662, 1987

Tracey KJ, Vlassara H, Cerami A: Cachectin/TNF (tumor necrosis factor). Lancet i: 1122, 1989

Tracey KJ: The acute and chronic pathophysiological effects of TNF: mediation of septic shock and wasting (cachexia). In: Beutler B, ed. Tumor necrosis factors: the molecules and their emerging role in medicine. New York: Raven Press, 255, 1992

Tracey KJ: TNF and Mae West or: death from too much of a good thing. Lancet 345: 75, 1995

Trauth BC, Klas C, Peters AMJ, Matzku S, Moller P, Falk W, Debatin K, Krammer PH: Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245: 301, 1989

Tsuchida H, Takeda Y, Takei H, Shinzawa H, Takahashi T, Sendo F: In vivo regulation of rat neutrophil apoptosis occurring spontaneously or induced with TNF-α or cyclohexamide. J Immunol 154: 2403, 1995

Van Antwerp DJ, Martin SJ, Kafri T, Green DR, Verma IM: Suppression of TNF-α-induced apoptosis by NFκB. Science 274: 787, 1996

Van der Poll T, Van Deventer SJ, Hack CE, Wolbink GJ, Aarden LA, Buller HR, Ten Cate JW: Effects on leukocytes after injection of tumor necrosis factor into healthy humans. Blood 79: 693, 1992

Van der Poll T, Calvano SE, Kumar A, Braxton CC, Coyle SM, Barbosa K, Moldawer LL, Lowry SF: Endotoxin induces downregulation of tumor necrosis factor receptors on circulating monocytes and granulocytes in humans. Blood 86: 2754, 1995

Van Ostade X, Vandenabeele P, Everaerdt B, Loetscher H, Brockhaus M, Lesslauer W, Tavernier J, Brouckaert P, Fiers W: Human TNF mutants with selective activity on the p55 receptor. Nature 361: 266, 1993

Van Ostade X, Vandenabeele P, Tavernier J, Fiers W: Human tumour necrosis factor mutants with preferential binding to and activity on either the R55 or R75 receptor. Eur J Biochem 220: 771, 1994

Van Otteren GM, Standiford TJ, Kunkel SL, Danforth JM, Strieter RM: Alterations of ambient oxygen tension modulate the expression of tumor necrosis factor and macrophage inflammatory protein-1 alpha from murine alveolar macrophages. Am J Respir Cell Mol Biol 13: 399, 1995

VanArsdale TL, VanArsdale SL, Force WR, Walter BN, Mosialos G, Kieff E, Reed JC, Ware CF: Lymphotoxin-beta receptor signaling complex: role of tumor necrosis factor receptor-associated factor 3 recruitment in cell death and activation of nuclear factor kappaB. Proc Natl Acad Sci USA 94: 2460, 1997

VanArsdale TL, Ware CF: TNF receptor signal transduction: ligand-dependent stimulation of a serine protein kinase activity associated with (CD120a) $TNFR_{60}$. J Immunol 153: 3043, 1994

Vandenabeele P, Declercq W, Vanhaesebroeck B, Grooten J, Fiers W: Both TNF receptors are required for TNF-mediated induction of apoptosis in PC60 cells. J Immunol 154: 2904, 1995

Vartio T, Seppa H, Vakeri A: Susceptibility of soluble and matrix fibronectin to degradation by tissue proteinases, mast cell chymase and cathepsin. J Biol Chem 256: 471, 1981

Vercellotti GM, Yin HQ, Gustafson KS, Nelson RD, Jacob HS: Platelet-activating factor primes neutrophil responses to agonists: role in promoting neutrophil-mediated endothelial damage. Blood 71: 1100, 1988

Verheji M, Bose R, Lin XH, Yao B, Jarvis WD, Grant S, Birrer MJ, Szabo E, Zon LI, Kyriakis JM, Haimovitz-Freidman A, Fuks Z, Kolesnick RN: Requirement for ceramide-initiated SAPK/JNK signaling in stress-induced apoptosis. Nature 380: 75, 1996

Verma IM, Stevenson JK, Schwartz EM, VanAntwerp D, Miyamoto S: Red/NF-κ/IκB family: intimate tales of association and dissociation.

Wallach D, Holtmann H, Engelmann H, Nopar Y: Sensitization and desensitization to lethal effects of tumor necrosis factor and IL-1. J Immunol 140: 2994, 1988

Wallach D: Cell induction by TNF: a matter of self control. TIBS 22: 107, 1997

Wang AM, Creasey AA, Ladner MB, Lin LS, Strickler J, Van Arsdell JN, Yamamoto R, Mark DF: Molecular cloning of the complementary DNA for human tumour necrosis factor. Science 228: 149, 1985

Wang CY, Mayo MW, Baldwin AS Jr: TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science 274: 784, 1996

Ware CF, Crowe PD, Grayson MH, Androlewicz MJ, Browning JL: Expression of surface lymphotoxin and tumour necrosis factor on activated T, B, and natural killer cells. J Immunol 149: 3881, 1992

Watson RWG, Redmond HP, Wang JH, Condron C, Bouchier-Hayes D: Neutrophils undergo apoptosis following ingestion of Escherichia coli. J Immunol 156: 3986, 1996

Weigmann K, Schutze S, Machleidt T, Witte D, Kronke M: Functional dichotomy of neutral and acidic sphingomyelinase in tumor necrosis factor signaling. Cell 78: 1005, 1994

Weisbart RH, Kwan L, Golde DW, Gasson JC: Human GM-CSF primes neutrophils for enhanced oxidative metabolism in response to the major physiological chemoattractants. Blood 69: 18, 1986

Weiss SJ, Peppin GJ: Collagenolytic metalloenzymes of the human neutrophil: characteristics, regulation and potential function in vivo. Biochem Pharmacol 35: 3189, 1986

Weiss SJ: Tissue destruction by neutrophils. New Engl J Med 320: 365, 1989

Weiss T, Grell M, Hessabi B, Bourteele S, Müller G, Scheurich P, Wajant H: Enhancement of TNF receptor p60-mediated cytotoxicity by TNF receptor p80: requirement of the TNF receptor-associated factor-2 binding site. J Immunol 158: 2398, 1997

Weitzman SA, Gordon LI: Inflammation and cancer: role of phagocyte-generated oxidants in carcinogenesis. Blood 76: 655, 1990

Whyte MKB: Ph D Thesis, University of London, 1993

Whyte MKB, Meagher LC, MacDermot J, Haslett C: Impairment of function in aging neutrophils is associated with apoptosis. J Immunol 150: 5123, 1993a

Whyte MKB, Meagher LC, Hardwick S, Savill J, Haslett C: Transient elevations of cytosolic free calcium retard subsequent apoptosis in neutrophils in vitro. J Clin Invest 92: 446, 1993b

Whyte MKB, Savill J, Meagher LC, Lee A, Haslett C: Coupling of neutrophil apoptosis to recognition by macrophages: Coordinated acceleration by protein synthesis inhibitors. J Leukoc Biol 62: 195, 1997

Wiley SR, Schooley K, Smolak PJ, Wenie SD, Chang-Pin H, Nicholl JK, Sutherland GR, Davis Smith T, Rauch C, Smith CA, Goodwin, RG: Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity 3: 673, 1995

Williams TJ: Role of neutrophils in reperfusion injury. In: The Handbook of immunology: Immunopharmacology of neutrophils (Hellewell PG and Williams TJ eds), Academic press, p245, 1994

Winterbourn CC, Van de Berg JJM, Roitman E, Kuypers FA: Chlorohydrin formation from unsaturated fatty acids reacted with hypochlorous acid. Arch Biochem Biophys 296: 547, 1992

Wolff SM, Clark RA, Root RK, Kimball HR: The Chediak-Higashi syndrome: studies in host defenses. Ann Int Med 76: 293, 1972

Worthen GS, Schwab B, Elson EL, Downey GP: Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. Science 245: 183, 1989

Wright SC, Zheng H, Zhong J, Torti FM, Larrick JW: Role of protein phosphorylation in TNF-induced apoptosis: phosphatase inhibitors synergize with TNF to activate DNA fragmentation in normal as well as TNF-resistant U937 variants. J Cell Biochem 53: 222, 1993

Wright SD, Kolesnick RN: Does endotoxin stimulate cells by mimicking ceramide? Immunology Today 16: 297, 1995

Wyllie AH, Kerr JFR, Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 68: 251, 1980

Wyllie AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555, 1980

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science 270: 1326, 1995

Xing Z, Kirpalani H, Torry D, Jordana M, Gauldie J: Polymorphonuclear leukocytes as a significant source of tumor necrosis factor-α in endotoxin-challenged lung tissue. Am J Pathol 143: 1009, 1993

Yanaga F, Watson SP: ceramide does not mediate the effect of tumour necrosis factor α on superoxide generation in human neutrophils. Biochem J 298: 733, 1994

Yao R, Cooper GM: Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 267: 2003, 1995

Yao R, Cooper GM: Growth factor-dependent survival of rodent fibroblasts requires phosphatidylinositol 3-kinase but is independent of $pp70^{S6K}$ activity. Oncogene 13: 343, 1996

Yee J, Christou NV: Neutrophil priming by lipopolysaccharide involves heterogeneity in calcium-mediated signal transduction. J Immunol 150: 1988, 1993

Yousefi S, Green DR, Blaser K, Simon HU: Protein tyrosine phosphorylation regulates apoptosis in human eosinophils and neutrophils. Proc Natl Acad Sci USA 91: 10868, 1994

Yuo A, Kitagawa S, Azuma E, Natori Y, Togawa A, Saito M, Takaku F: Tyrosine phosphorylation and intracellular alkalinization are early events in human neutrophils stimulated by tumour necrosis factor, granulocyte macrophage colony-stimulating factor and granulocyte colony-stimulating factor. Biochim Biophys Acta 1156: 197, 1993

Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B, Kroemer G: Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182: 367, 1995

Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G: Mitochondrial control of nuclear apoptosis. J Exp Med 183: 1533, 1996a

Zamzami N, Marchetti P, Castedo M, Hirsch T, Susin SA, Masse B, Kroemer G: Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. FEBS Letts 384: 53, 1996b

Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ: Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377: 348, 1995

Zigmond SH, Hirsch JG: Effects of cytochalasin B on polymorphonuclear leukocyte locomotion, phagocytosis and glycolysis. Exp Cell Res 73: 383, 1972

Zu YL, Ai Y, Gilchrist A, Labadia ME, Sha'afi RI, Huang CK: Activation of MAP kinase-activated protein kinase 2 in human neutrophils after phorbol ester or FMLP peptide stimulation. Blood 87: 5287, 1996

Regulation of Neutrophil Apoptosis by Tumor Necrosis Factor-α: Requirement for TNFR55 and TNFR75 for Induction of Apoptosis In Vitro

By Joanna Murray, Jeffrey A.J. Barbara, Sarah A. Dunkley, Angel F. Lopez, Xaveer Van Ostade, Alison M. Condliffe, Ian Dransfield, Christopher Haslett, and Edwin R. Chilvers

Granulocyte apoptosis is an important mechanism underlying the removal of redundant neutrophils from an inflammatory focus. The ability of many proinflammatory agents to impede this event suggests that such agents act not only in a priming or secretagogue capacity but also increase neutrophil longevity by delaying apoptosis. We have examined whether this hypothesis holds true for all neutrophil priming agents, in particular tumor necrosis factor-α (TNF-α), which has been variably reported to either induce, delay, or have no effect on neutrophil apoptosis. After 20 hours coincubation TNF-α inhibited neutrophil apoptosis; however, more detailed analysis demonstrated its ability to promote apoptosis in a subpopulation of cells at earlier (2 to 8 hours) times. Formyl-Met-Leu-Phe, platelet-activating factor, inositol hexakisphosphate, lipopolysaccharide, leukotriene B_4, and granulocyte-macrophage colony-stimulating factor all inhibited apoptosis at 6 and 20 hours. The early proapoptotic effect of TNF-α was concentration-dependent (EC_{50} 2.8 ng/mL), abolished by TNF-α neutralizing antibody, and was not associated with any change in cell viability or recovery. Of relevance to the inflamed site, the ability of TNF-α to accelerate apoptosis was lost if neutrophils were primed with 1 μmol/L PAF or aged for 6 hours before TNF-α addition. The TNFR55-selective TNF-α mutants (E146K, R32W-S86T) induced neutrophil apoptosis but with a potency 14-fold lower than wild-type TNF-α. Although the TNFR75-selective mutant (D143F) did not induce apoptosis, blocking antibodies to both receptor subtypes abolished TNF-α–stimulated apoptosis. Hence, TNF-α has the unique ability to induce apoptosis in human neutrophils via a mechanism where TNFR75 facilitates the dominant TNFR55 death effect. This may be an important mechanism controlling neutrophil longevity and clearance in vivo.

NEUTROPHIL APOPTOSIS, which results in the recognition and uptake of these cells by macrophages, has been proposed as an important mechanism for the removal of neutrophils from sites of inflammation.[1-6] Experimental data obtained using human peripheral blood neutrophils show that these cells undergo constitutive apoptosis when aged in vitro and that this process is associated with maintenance of membrane integrity, hyporesponsiveness to external secretagogue signals, and the capacity to be phagocytosed intact by macrophages and certain other cells with phagocytic potential.[1-5,7] The speed and capacity of the macrophage phagocytic response toward apoptotic neutrophils, together with the observation that engulfment does not excite a proinflammatory macrophage response,[8] predicts that this process plays an important role in the safe disposal of intact but effete neutrophils from an inflamed focus. This view is supported by the recent demonstration of this process occurring in vivo; for example, in endotoxin-induced experimental lung injury,[9] the neonatal respiratory distress syndrome,[10] and experimental glomerulonephritis.[11]

Although the mechanisms involved in regulating neutrophil survival and death are poorly understood, there is now considerable evidence to suggest that this process is not immutable because the rate at which these cells undergo apoptosis, at least in vitro, can be altered. For example, the colony-stimulating factors granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage-CSF (GM-CSF), lipopolysaccharide (LPS), and hypoxia all increase the survival of these cells by delaying apoptosis[12-17] implying that certain agents may upregulate neutrophil function both by priming for enhanced functional responsiveness to secretagogue agonists and by delaying apoptosis which prolongs their functional life span.[16]

One cytokine of particular interest in this paradigm is tumour necrosis factor-α (TNF-α). This agent is a powerful priming agonist in neutrophils yet has the capacity along with other low-affinity nerve growth factor (NGF) receptor ligands to induce apoptosis in a variety of cells, including T lymphocytes[18] and HL-60 cells.[19] In contrast, a number of reports have indicated that TNF-α has the opposite effect, ie, delays apoptosis, in human monocytes, neutrophils, and B104 lymphoma cells.[20-22] Interest in this area has been heightened by the recent finding of Zheng et al,[18] who showed that the induction of apoptosis in $CD8^+$ T cells by TNF-α is mediated via the TNFR75 rather than the classical death domain–containing TNFR55.[23]

The aim of this study was to undertake a detailed characterization of the effects of TNF-α on neutrophil apoptosis in vitro. We show that while prolonged incubation (>12 hours) of human neutrophils with TNF-α indeed causes a decrease in the extent of apoptosis, TNF-α can induce apoptosis in a proportion of cells at earlier times (<8 hours). The early proapoptotic effect of TNF-α (1) appears to be unique to this cytokine because it is not observed with other priming or secretagogue agonists, (2) is abolished by initial priming of the cells with platelet-activating factor (PAF), an

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effect that is not secondary to a change in TNFR55 or TNFR75 expression, and (3) appears critically dependent on TNF-α interaction with both TNF receptor subtypes as determined by blocking TNF-α receptor antibodies and TNF-α mutants which display selective binding to the two TNF-α receptors.[24-26]

MATERIALS AND METHODS

Neutrophil preparation and culture. Human neutrophils were purified from the peripheral blood of healthy human volunteers exactly as detailed previously.[27] Cell purity was assessed using cytocentrifuge preparations fixed in methanol and stained with May/Grünwald/Giemsa (Merck Ltd, Lutterworth, Leicestershire, UK) and was routinely greater than 95% neutrophils with less than 0.1% mononuclear cell contamination. Viability was assessed by the ability of the cells to exclude trypan blue and was consistently greater than 99% under all experimental conditions reported. Freshly harvested neutrophils were routinely suspended at a density of 5×10^6/mL in Iscove's modified Dulbecco's medium (MDM) supplemented with 10% autologous serum and 50 U/mL penicillin and streptomycin and cultured at 6.75×10^5 cells/150 μL in flat-bottomed 96-well Falcon flexiwell plates (Becton Dickinson, Oxford, UK) in a humidified 5% CO_2 atmosphere at 37°C.

To examine the effects of neutrophil priming agents, freshly isolated cells were cultured in the presence or absence of (final concentrations) formyl-Met-Leu-Phe (fMLP) (1 nmol/L), PAF (1 μmol/L), inositol hexakisphosphate ($InsP_6$; 100 μmol/L), LPS (100 ng/mL), leukotriene B_4 (LTB_4; 100 nmol/L), GM-CSF (50 U/mL), or TNF-α (0.005 to 100 ng/mL). After incubation for the time periods indicated (1 to 20 hours), apoptosis was quantified as detailed below.

Morphological assessment of PMN apoptosis. After gentle resuspension, neutrophils were harvested, cytocentrifuged, and the resulting slide preparations fixed and stained as described above with cell viability (trypan blue exclusion) and recovery assessed in parallel. Cell morphology was examined under oil immersion light microscopy, and apoptotic neutrophils defined as cells containing darkly stained pyknotic nuclei.[1] For each condition examined, slides were prepared from triplicate incubations and a total of at least 500 neutrophils covering five high-power fields counted with the observer blinded to the assay conditions.

Chromatin fragmentation assay and propidium iodide staining. For preparation of genomic DNA, neutrophils were incubated in the presence or absence of 1 to 100 ng/mL TNF-α or 10 and 100 ng/mL E146K and D143F for 3 hours, pelleted, and resuspended in 2 mL 150 mmol/L sodium acetate, 5 mmol/L EDTA (pH 7). After addition of a further 2 mL of sodium acetate/EDTA containing 0.2 mg/mL proteinase K and 1% sodium dodecyl sulphate (SDS) the

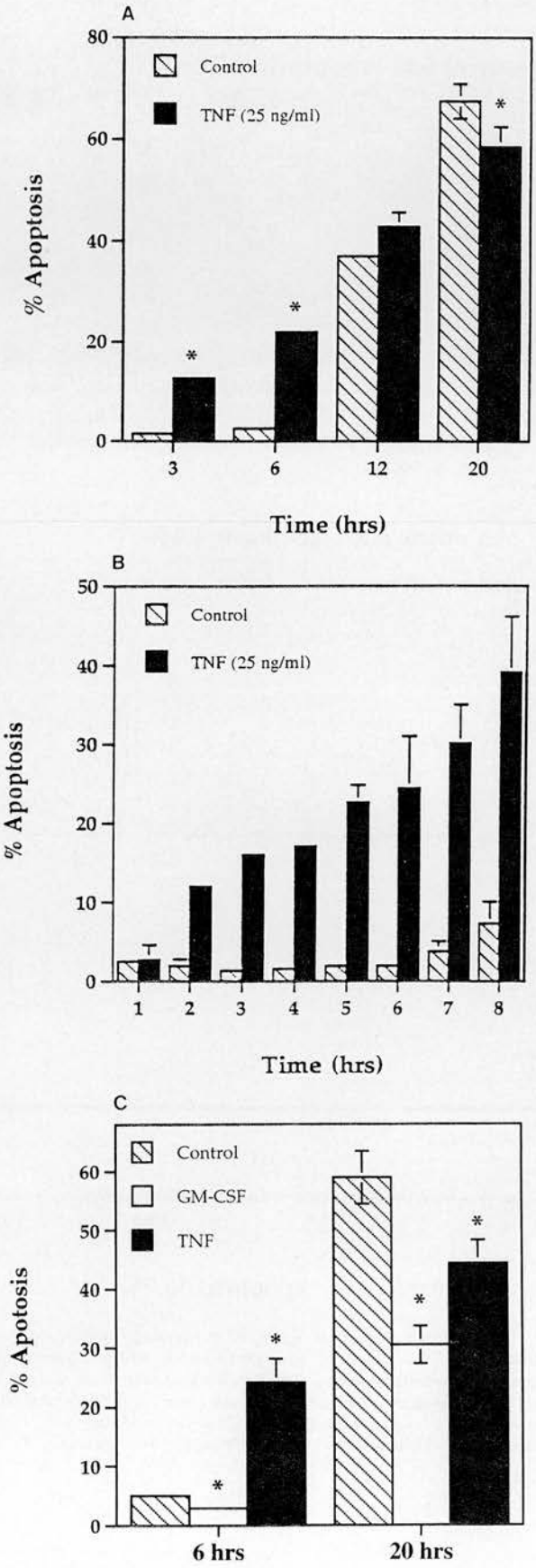


Fig 1. Time course for the effect of TNF-α on apoptosis in human neutrophils. Human neutrophils (5×10^6/mL) were incubated in MDM containing 10% autologous serum alone (▨), or in the presence of 25 ng/mL TNF-α (■) or 50 U/mL GM-CSF (□) at 37°C. At the time periods indicated, the cells were resuspended and cytocentrifuge preparations made. These were fixed, stained, and percent apoptosis assessed morphologically. (A) Represents the effects of TNF-α after 3, 6, 12, and 20 hours of incubation with the results from a separate series of experiments over a 1- to 8-hour time course shown in (B). The comparative effects of GM-CSF on neutrophil apoptosis at 6 and 20 hours are shown in (C). All values represent mean ± SEM of n = 3 separate experiments, each performed in triplicate (*$P < .05$ compared with time-matched controls). Where not shown, SEM values are less than 2% of means.

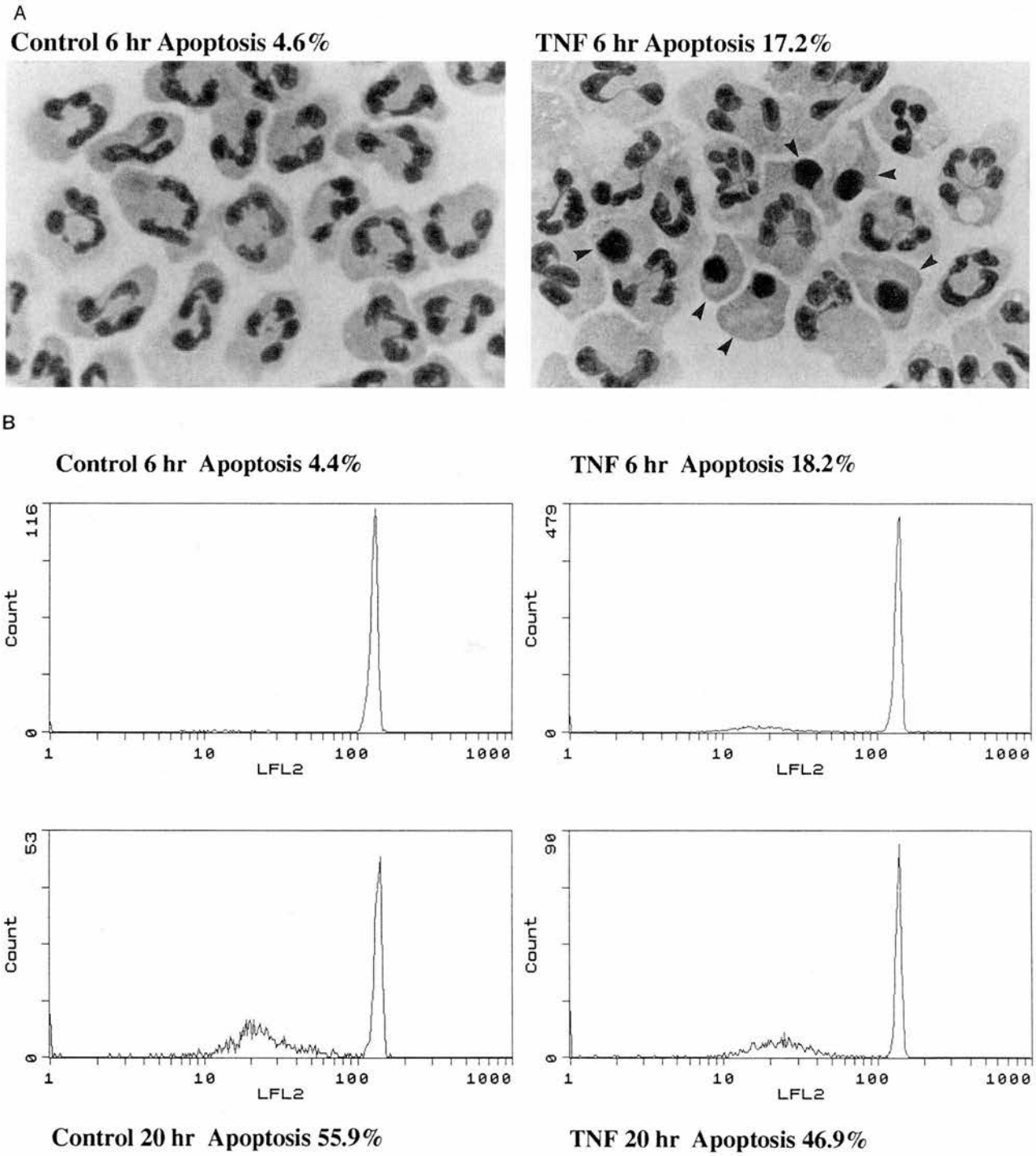


Fig 2. Effect of TNF-α on neutrophil morphology and propidium iodide (PI) staining. Human neutrophils were incubated in the presence (A and B, righthand panels) or absence (A and B, lefthand panels) of 12.5 ng/mL TNF-α as detailed in the legend to Fig 1. (A) Cytocentrifuge preparations were prepared after 6 hours and cells fixed, stained, and examined under 100× objective oil immersion light microscopy. Cells with classical apoptotic morphology are arrowed. (B) After 6 or 20 hours in culture cells were resuspended in ice-cold 70% ethanol, washed in PBS, and incubated with PI in the presence of RNAase before analysis using an EPICS Profile II. Mean fluorescence values are shown for a minimum of 5,000 cells for each condition and are representative of six to nine determinations in three separate experiments.

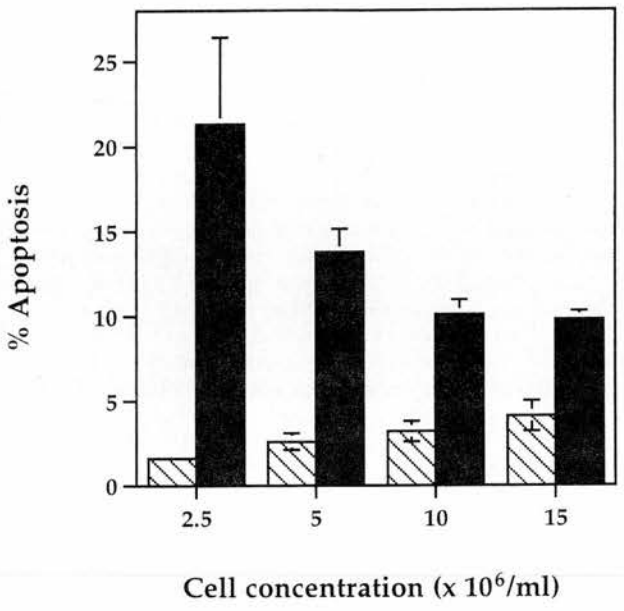


Fig 3. Effect of neutrophil cell concentration on the early proapoptotic effect of TNF-α. Human neutrophils were incubated at a cell concentration of 2.5 to 15 × 10⁶/mL in the presence (■) or absence (▨) of TNF-α (12.5 ng/mL) for 6 hours at 37°C. Cytocentrifuge slides were then prepared for morphological assessment of apoptosis. Data represent the mean ± SEM of six observations in two separate experiments.

samples were incubated overnight at 55°C. Fresh proteinase K (0.1 mg/mL) was added and samples were incubated for a further 1 hour at 55°C before cooling to room temperature. Thereafter, 4 mL of phenol was added, samples mixed for 30 minutes, centrifuged (5,000*g*, 10 minutes), and the DNA phase extracted in an equal volume of phenol/chloroform. After centrifugation the upper phase was removed and extracted in an equal volume of chloroform and the DNA precipitated with 2 vol of absolute ethanol. The precipitate was washed in 75% ethanol followed by absolute ethanol. After evaporation of the ethanol at room temperature the pellet was dissolved in 250 mL TE buffer (10 mmol/L Tris/HCl, 1 mmol/L EDTA, pH 8) and quantified by reading the absorbance at 260 nm (DU-50 Beckman spectrophotometer, High Wycombe, UK). Loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) containing RNAase A (1:100 dilution of 10 mg/mL) was added and 3 to 10 μg DNA electrophoresed in a 1.2% agarose gel at 40 V in 40 mmol/L Tris-acetate, 1 mmol/L EDTA.

Propidium iodide (PI) staining was assessed according to Nicoletti et al,[28] with the following modifications: 0.4 × 10⁶ cells were suspended in 100 μL ice-cold 70% ethanol for 10 minutes, washed (×3) in phosphate-buffered saline (PBS) (4°C), and resuspended in 30 μL PBS and 30 μL RNAase (100 μg/mL). After gentle mixing 60 μL of propidium iodide (100 μg/mL in PBS) was added; the cells were incubated in the dark at room temperature for 15 minutes and after washing in PBS stored at 4°C before analysis using an EPICS Profile II (Coulter Electronics, Luton, UK). Mean fluorescence values were determined from a minimum of 5,000 cells within an analysis region corresponding to nonfragmented neutrophils.

Measurement of superoxide anion generation. The release of superoxide anions was determined by means of the superoxide dismutase-inhibitable reduction of cytochrome C. Neutrophils (10^6 in 90 μL PBS containing $CaCl_2$ and $MgCl_2$) were placed in 2-mL polypropylene tubes containing 10 μL TNF-α to give a final concentration of 0.05 to 50 ng/mL, or PBS (control). Each reaction was performed in quadruplicate. Samples were incubated in the presence or absence of TNF-α for 30 minutes at 37°C in a shaking water bath before the addition of 750 μL cytochrome C (final concentration 1.2 mg/mL) to all samples and 50 μL of superoxide dismutase (20 μg/mL in PBS) to one sample in each quadruplicate. After a final 10-minute incubation with PBS or fMLP (100 nmol/L), samples were placed on ice and the optical density of the supernatants determined using a scanning spectrophotometer (Pye-Unicam 8700; Unicam Ltd, Cambridge, UK) measuring peak height at 550 nm with the paired dismutase-containing supernatants as reference. Superoxide release (nmoles/10^6 cells) was calculated using the extinction coefficient 21 × 10^3 $(mol/L)^{-1} \cdot cm^{-1}$.

Effects of TNF-α neutralizing antibodies, TNFR55 and TNFR75 blocking antibodies, and TNF-α receptor-selective mutants on basal and TNF-α–stimulated neutrophil apoptosis. Freshly isolated neutrophils were cultured as detailed above in either MDM plus 10% autologous serum alone (control), TNF-α (12.5 ng/mL), mouse IgG_1 antihuman TNF-α neutralizing monoclonal antibody (MoAb) (1 μg/mL), or an identical concentration of TNF-α preincubated for 90 minutes at 37°C with the neutralizing antibody. At 6 hours cells were harvested and apoptosis assessed as indicated above. To assess the effect of TNFR55 and TNFR75 blocking antibodies on the ability of TNF-α to modulate the rate of neutrophil apoptosis, cells (5 × 10^6/mL) were preincubated for 30 minutes at 37°C in MDM containing 10% autologous serum in the presence or absence of 28 μg/mL of mouse IgG_1 antihuman TNFR55, rat IgG_{2b} antihuman TNFR75, or isotype-matched IL-2α (p55) receptor MoAb before a 6- or 20- hour incubation with TNF-α (12.5 ng/mL) or buffer and morphological assessment of apoptosis. The saturating concentration of TNFR75 MoAb used was predetermined using a flow cytometry antibody titration assay[29] and TNFR55 MoAb used at an identical concentration. In experiments using the TNFR55-selective mutants E146K and R32W-S86T or TNFR75-selective mutant D143F, freshly isolated neutrophils were incubated for 3 or 6 hours in the presence or absence of 0.01 to 100 ng/mL mutein or rhTNF-α as previously detailed[30] before morphological quantification of apoptosis or examination of DNA fragmentation.

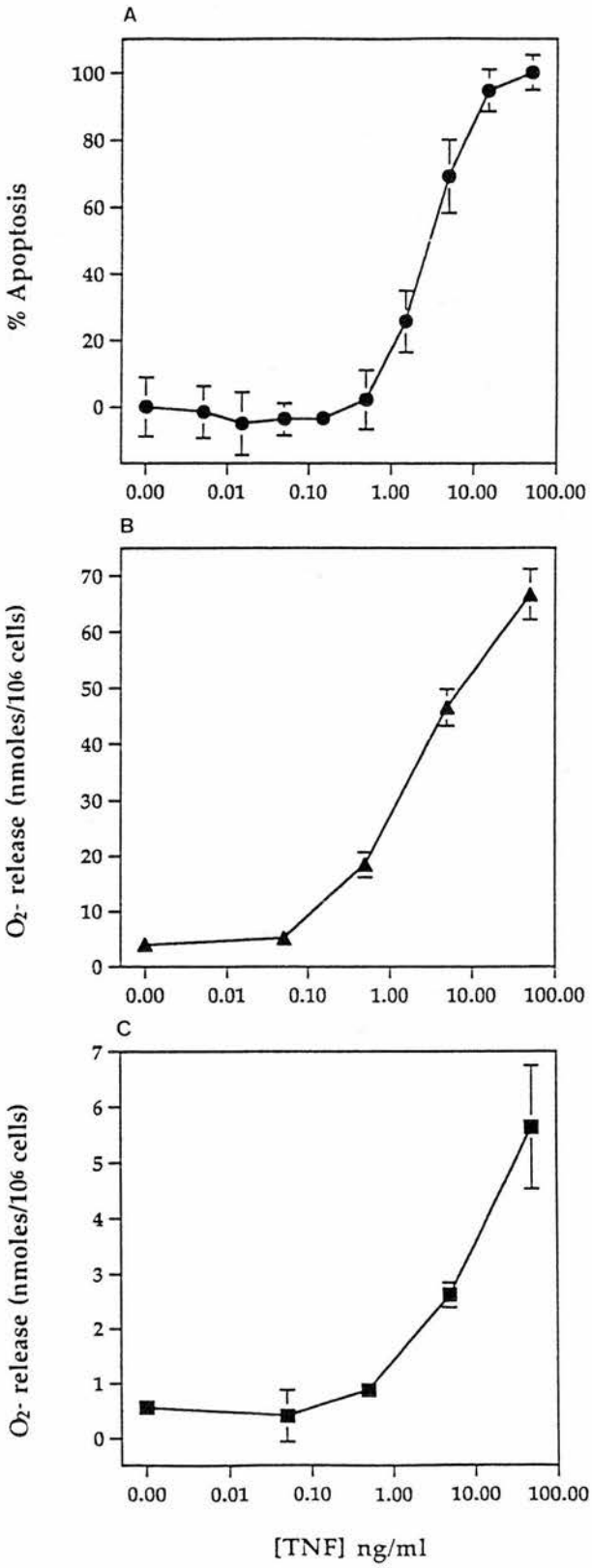
Flow cytometric analysis of TNFR55 and TNFR75 expression. Neutrophils were prepared, preincubated for 5 minutes with 1 μmol/L PAF or buffer, and then incubated (6.75 × 10^5 cells/150 μL MDM containing 10% autologous serum) in flexiwell plates at 37°C for 0 to 6 hours as detailed above. At the appropriate time points the cells were transferred to prechilled U-bottomed flexiwell plates,

Table 1. Effect of Incubation Time and Neutrophil Presence on the Concentration of TNF-α in Supernatants

Time (h)	Medium + TNF-α (ng/mL)	Neutrophils + TNF-α (ng/mL)	Neutrophils − TNF-α (ng/mL)
0	8.87 ± 0.59	10.29 ± 1.10	0*
3	8.33 ± 0.54	7.84 ± 0.62	0
6	9.18 ± 0.90	8.67 ± 0.97	0
12	8.96 ± 1.08	8.29 ± 0.68	0
20	8.69 ± 0.63	7.97 ± 0.67	0

Human neutrophils (5 × 10^6/mL) or medium alone (MDM containing 10% autologous serum) were incubated in the presence (+TNF-α) or absence (−TNF-α) of 12.5 ng/mL TNF-α in flexiwell plates in an humidified 5% CO_2 incubator at 37°C for 0 to 20 hours. The supernatants were harvested, frozen, and immunoreactive human TNF-α quantified by ELISA as detailed in the Materials and Methods section. Data represent mean ± SEM of six measurements performed in two separate experiments.

* Assay sensitivity 15.6 pg/mL.



washed with ice-cold PBS containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide, and resuspended in 40 μL of a saturating concentration of mouse-antihuman TNFR55, TNFR75, or CD2 MoAb as a negative control (UCHT-1 clone, IgG_1; SAPU, Carluke, UK). After a 30-minute period on ice the cells were washed twice and incubated with 40 μL fluorescein isothiocyanate (FITC)-conjugated goat-antimouse Ig (Dako, Buckinghamshire, UK; diluted 1 in 40 with PBS/BSA/azide buffer). After washing, samples were analyzed using an EPICS Profile II (Coulter Electronics) and mean fluorescence values from a minimum of 3,000 cells determined.

Enzyme-linked immunosorbent assay (ELISA) for TNF-α. Extracellular immunoreactive human TNF-α was quantified using a modification of the double-ligand method as previously described.[31] In brief, flat-bottomed 96-well microtiter plates (Immulon 1; Dynatech, Billinghurst, UK) were coated with 100 μL/well mouse-antihuman TNF-α MoAb (2.5 μg/mL in coating buffer; Dynatech) for 3 hours at 37°C and then washed with PBS (pH 7.5), 0.05% Tween-20 (PBS-Tween-20). Microtiter plate nonspecific binding sites were blocked with Dynatech blocking buffer (30 minutes, 37°C). The plates were washed four times (PBS-Tween-20) and cell-free supernatants or TNF-α standards (half-log dilutions of recombinant human [rh]TNF-α, from 7.8 to 500 pg/mL) added in 100-μL aliquots in duplicate and incubated for 16 hours at 4°C. Plates were washed four times (PBS-Tween-20), followed by the addition of 100 μL/well rabbit polyclonal antihuman TNF-α antibody (1 μg/mL in assay diluent; Dynatech) and the plates were incubated for 3 hours at room temperature. The plates were again washed, alkaline phosphatase–conjugated donkey-antirabbit IgG added, and following a further incubation of 3 hours at room temperature, p-nitrophenyl phosphate substrate added in 10% diethylamine (DEA) buffer. Plates were read at 410 nm in an ELISA reader.

Statistics. All values are presented as the mean ± SEM of (n) number of independent experiments. The data were evaluated statistically using the paired or unpaired Students *t*-test: P values $<.05$ were considered to be statistically significant.

Materials. Recombinant human TNF-α (10 μg/mL in PBS) and GM-CSF (1 μg/mL in PBS) were purchased from Genzyme (Cambridge, MA) and stored at −70°C before use. Rat-antihuman TNFR75 MoAb and interleukin-2 (IL-2) receptor MoAb were also purchased from Genzyme (Cambridge, MA) and stored at 0.4 to 1 mg/mL in PBS (4°C). The mouse IgG_1 antihuman TNF-α neutralizing MoAb and mouse-antihuman TNFR55 and TNFR75 MoAbs were purchased from R & D Systems Europe (Abingdon, Oxon, UK). LTB_4, LPS (*Escherichia coli* 0111:B4, γ-irradiated), $InsP_6$ (dipotassium salt), fMLP, superoxide dismutase, cytochrome C, Dulbecco's PBS (with or without $CaCl_2$ and $MgCl_2$), p-nitrophenyl phosphate, proteinase K, trypan blue, and dextran-500 were all purchased from Sigma Chemical Co (Poole, Dorset, UK). fMLP was dissolved in sterile dimethyl sulfoxide (DMSO) at a stock concentration of 1 mmol/L and stored at −20°C. LTB_4 was stored in ethanol at 100 μg/mL at −20°C. C18 PAF was obtained from Calbiochem-Novabiochem UK Ltd (Nottingham, UK) and dissolved in ethanol

Fig 4. Concentration-response relationships for TNF-α–induced apoptosis, priming, and direct superoxide anion generation. Human neutrophils were prepared and incubated at 5 × 10^6/mL with 0 to 100 ng/mL TNF-α for 6 hours for assessment of apoptosis (A) or at 10^7/mL with 0 to 100 ng/mL TNF-α for 30 minutes before assessment of fMLP-stimulated (100 nmol/L, 10 minutes) (B) or direct superoxide anion generation (10 minutes) (C) using a cytochrome C reduction assay (see Materials and Methods). Values represent mean ± SEM of n = 3 separate experiments, each performed in triplicate. Where not shown, SEM values are less than 2% and fall within the symbols.

at a stock concentration of 10 mmol/L. Percoll was obtained from Pharmacia Biotech (St Albans, Hertfordshire, UK). MDM was obtained from Life Technologies (Paisley, Scotland, UK). Alkaline phosphatase–conjugated donkey-antirabbit IgG was purchased from Stratech Scientific Ltd (Luton, Bedfordshire, UK). All other reagents were obtained from Sigma Chemical Co or Merck Ltd and were of the highest purity available.

RESULTS

Effect of TNF-α on the rate of neutrophil apoptosis in vitro. In agreement with previous studies,[21] coincubation of human neutrophils with 25 ng/mL TNF-α for 20 hours caused a small but significant inhibition of neutrophil apoptosis (Fig 1A and C). This effect concurs with our previous observations that many other agents that prime or activate human neutrophils either delay (GM-CSF, Fig 1C, C5a, LPS,[16] $InsP_6$,[32] and LTB_4, results not shown), or have no effect on (fMLP and PAF, results not shown), the rate of constitutive apoptosis when assessed at this time point. However, when cytospins were prepared at earlier times (3 to 6 hours) when the basal rate of apoptosis was still low, TNF-α caused a significant increase in the number of apoptotic cells present (Fig 1A). This early proapoptotic effect of TNF-α was rapid (observed in all experiments by 2 hours, Fig 1B), maximal in terms of the ratio of apoptotic to nonapoptotic cells by 6 hours (Fig 1B), and was not observed with any of the other priming/activating agents tested at 6 hours (50 U/mL GM-CSF [Fig 1C], 1 and 100 nmol/L fMLP, 1 μmol/L PAF, 100 μmol/L $InsP_6$, or 100 μmol/L LTB_4, data not shown). Accurate quantification of the effects of such agents (including TNF-α) beyond 24 hours was not possible because of the development of secondary necrosis at these times.

The induction of apoptosis by TNF-α at early time points was confirmed by DNA electrophoresis, which showed a typical ladder pattern in TNF-α–treated neutrophils indicative of endonuclease-dependent internucleosomal cleavage (see Fig 8), and by PI staining, which revealed an increase in the percentage of cells with hypodiploid DNA content in TNF-α–treated cells (Fig 2B) (control 6 hours, 4.4% ± 0.3%; 12.5 ng/mL TNF-α 6 hours, 18.2% ± 0.1%; $P < .05$, n = 3). These latter values exactly match the extent of apoptosis assessed by morphological criteria (Fig 2A) (control 6 hours, 4.6% ± 0.2%; 12.5 ng/mL TNF-α 6 hours, 17.2% ± 1.4%; $P < .05$, n = 3). The late inhibitory effect of TNF-α on neutrophil apoptosis (20 hours) was also confirmed using this technique (Fig 2B) (percent low PI stained cells: control 20 hours, 55.9% ± 0.3%; 12.5 ng/mL TNF-α 20 hours, 46.9% ± 1.5%; $P < .05$, n = 3).

The specificity of action of TNF-α was shown in experiments where the proapoptotic effect of TNF-α was completely abolished by preincubation with mouse-antihuman TNF-α neutralizing antibody (percent apoptosis at 6 hours: control, 6.7% ± 0.6%; 25 ng/mL TNF-α, 17.3% ± 1.4%; TNF-α antibody, 6.2% ± 0.8%; TNF-α + TNF-α antibody, 6.0 ± 0.7%; n = 3). In addition, TNF-α did not affect neutrophil viability (trypan blue exclusion) or recovery rates (data not shown), excluding the possibility that the increased rates of early apoptosis observed reflected either TNF-α–induced adhesion of nonapoptotic cells[33] or a TNF-α–induced switch between necrosis and apoptosis as seen in human leukemic cells treated with high concentrations of alkylating agents.[34] Examination of the influence of neutrophil cell concentration

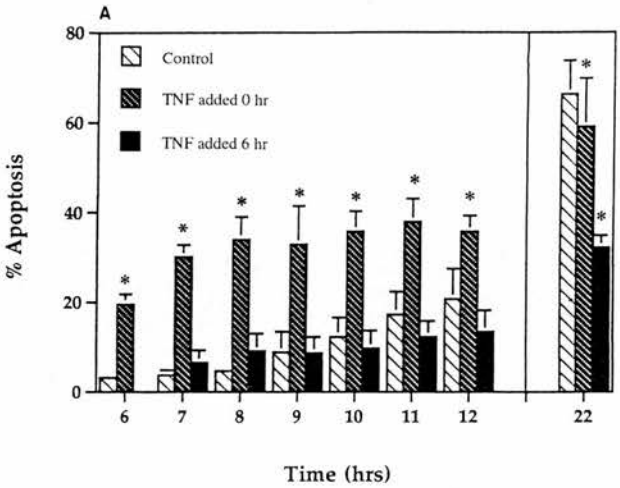


Fig 5. Loss of the early proapoptotic effect of TNF-α in human neutrophils after delayed addition of TNF-α or pretreatment with PAF. (A) To assess the effect of delayed addition of TNF-α, human neutrophils were incubated at 5 × 10^6/mL in the absence (▨) or presence of 12.5 ng/mL TNF-α added either at the beginning of the incubation period (▨) or after a delay of 6 hours (■). The extent of apoptosis was then assessed morphologically at the time points indicated. All data values represent the mean ± SEM of n = 3 separate experiments, each performed in triplicate (*$P < .05$ compared with time-matched controls). Where not shown, SEM values are less than 2% of means. (B) Human neutrophils (5 × 10^6/mL) were incubated in MDM containing 10% autologous serum in the presence or absence of PAF (1 μmol/L) for 5 minutes before being placed in 96-well flexiwell plates for a 6-hour incubation with 12.5 ng/mL TNF-α or buffer. Apoptosis was then determined on cytocentrifuge preparations using standard morphological criteria. Values represent mean ± SEM of three separate experiments, each performed in triplicate (*$P < .05$ compared with controls).

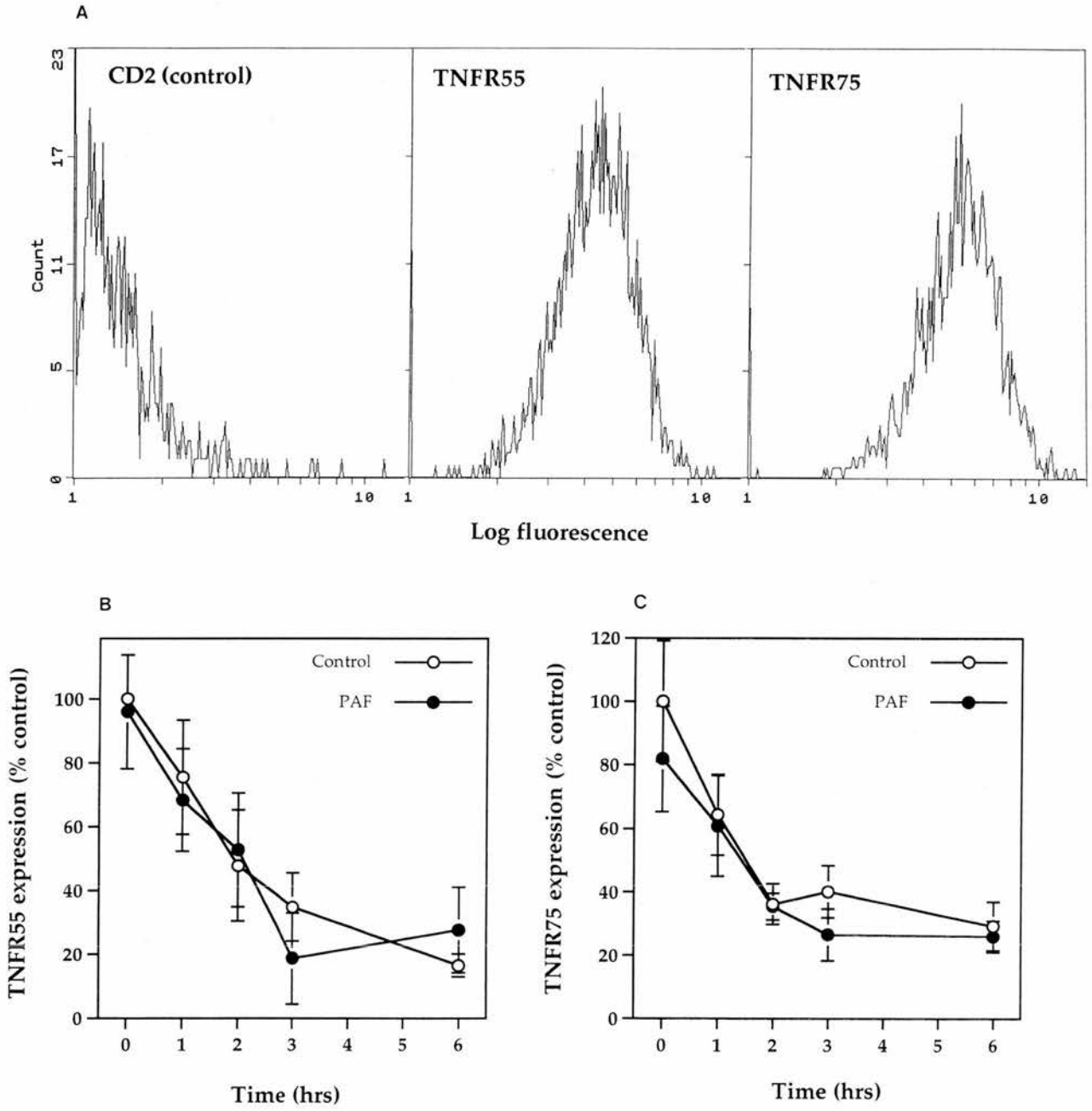


Fig 6. Time-course for TNFR55 and TNFR75 expression in the presence and absence of PAF. (A) Flow cytometric analysis of CD2 (negative control), TNFR55, and TNFR75 receptor expression in freshly isolated neutrophils. Histograms represent profiles of cell count against log fluorescence and are representative examples from nine separate experiments. (B and C) Human neutrophils (5×10^6/mL) were incubated in MDM containing 10% autologous serum in the presence or absence of PAF (1 μmol/L) for 5 minutes before incubation for 1 to 6 hours in flexiwell plates at 37°C. At these time points TNFR55 (B) and TNFR75 (C) expression was quantified by flow cytometry as detailed in Materials and Methods. Data represent percent of specific receptor expression values obtained in freshly isolated cells with each point being the mean ± SEM of three separate experiments, each performed in triplicate.

on the action of TNF-α at 6 hours showed that the proapoptotic effect was most marked at 2.5 to 5×10^6 cells/mL (Fig 3). A cell concentration of 5×10^6/mL was selected for use in subsequent studies as this was the optimal cell density for preparation and quantification of cytospins. Assay of the TNF-α concentration present in cell supernatants at 0, 6, and 20 hours showed no evidence of spontaneous TNF-α production[35] or decline in the level of exogenously added TNF-α (Table 1).

Relationship between TNF-α–induced apoptosis and priming. The potential physiological relevance of the proapoptotic effect of TNF-α was assessed initially by compar-

ing the concentration-dependency of this response with the ability of TNF-α to directly stimulate superoxide anion generation in neutrophils and to prime fMLP (100 nmol/L)-stimulated respiratory burst activity. Figure 4 shows the very similar concentration-response relationships for all three effects with an EC_{50} for TNF-α–induced apoptosis of 2.8 ng/mL.

To assess whether the induction of apoptosis by TNF-α was itself influenced by neutrophil priming, cells were either preincubated in flexiwell plates for 6 hours or pretreated with 1 μmol/L PAF for 5 minutes before the addition of TNF-α. Priming with PAF resulted in a dramatic upregulation of fMLP-stimulated superoxide anion release (superoxide anion release, nmol/10^6 cells/10 min, control 0.9 ± 0.1, 100 nmol/L fMLP 3.4 ± 0.4, 1 μmol/L PAF 0.8 ± 0.1, 1 μmol/L PAF + 100 nmol/L fMLP 33.1 ± 2.0, n = 3) with the adhesion-based model also converting TNF-α into a full secretagogue agonist[33,36] (and data not shown). As shown in Fig 5, both strategies abolished the proapoptotic effect of TNF-α, although by contrast, the late inhibitory effect of TNF-α on apoptosis at 20 hours appeared to be enhanced when TNF-α addition was delayed (Fig 5A). Flow cytometric analysis of TNFR55 and TNFR75 expression in freshly isolated neutrophils demonstrated a single population of TNFR55 and TNFR75 positive cells (Fig 6A) with mean fluorescence values of 4.06 ± 0.26 and 5.40 ± 0.53, respectively (CD2 control values 1.50 ± 0.10, n = 9). Incubation of neutrophils in flexiwell plates under the identical conditions described above resulted in a time-dependent decrease in both TNFR55 and TNFR75 expression (Fig 6B and C); however, preincubation of neutrophils with 1 μmol/L PAF for 5 minutes, which completely abolished the ability of TNF-α to induce apoptosis, had no effect on either TNFR55 or TNFR75 expression (Fig 6B and C).

TNFR55 and TNFR75 dependency of TNF-α–stimulated neutrophil apoptosis. The involvement of TNFR55 and TNFR75 in mediating the early proapoptotic effect of TNF-α was examined using TNF-α receptor subtype selective blocking MoAbs and agonistic mutants. In preliminary experiments the dominant role of TNFR55 in mediating TNF-α–induced priming of superoxide anion generation in neutrophils in suspension was confirmed[37,38] in that the TNFR55 MoAb completely blocked TNF-α–induced priming of fMLP-stimulated superoxide anion generation whereas the TNFR75 MoAb had no effect on this response (data not shown). In contrast, however, an identical 30-minute preincubation of neutrophils with either TNFR55 or TNFR75 MoAb completely abrogated the proapoptotic effect of TNF-α (Fig 7). Neither the antibodies themselves (Fig 7) nor isotype-matched control MoAbs (data not shown) affected basal or, in the latter case, TNF-α–stimulated apoptosis. Of interest, the TNFR75 MoAb did not influence the late inhibitory effect of TNF-α on apoptosis observed at 20 hours (percent apoptosis at 20 hours: control, 58.7% ± 1.2%; TNF-α, 38.7% ± 3.4%; TNFR75 MoAb, 51.4% ± 1.6%; TNF-α + TNFR75 MoAb, 32.4% ± 1.0%). These data indicate that the TNF-α–induced priming and late inhibitory effect on apoptosis are mediated via TNFR55, but the early proapoptotic effect is dependent on both TNFR55 and TNFR75 activation.

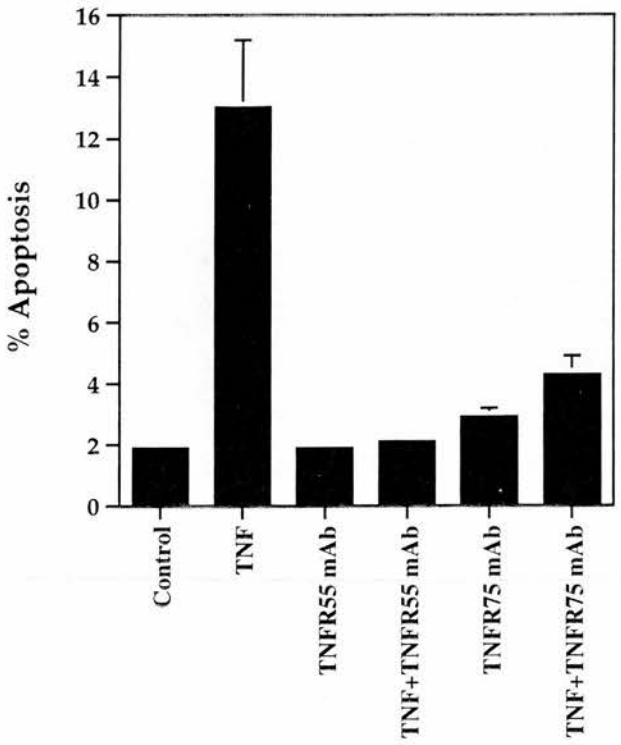
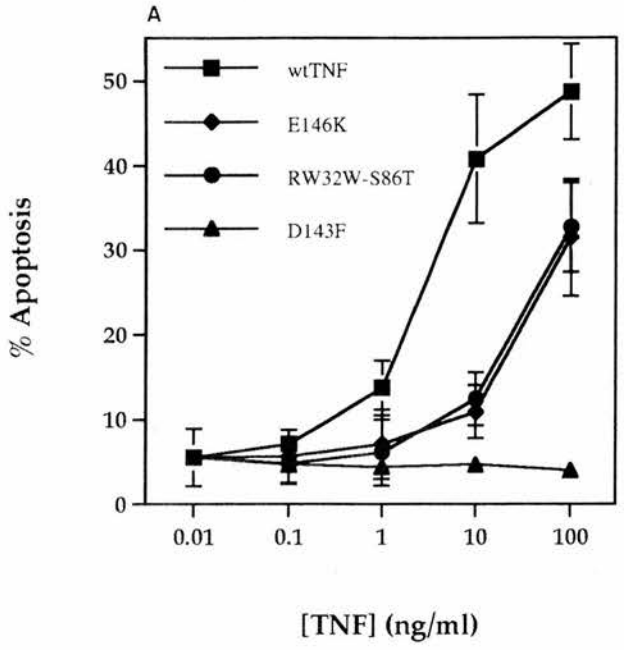


Fig 7. Effect of TNFR55 and TNFR75 blocking antibodies on TNF-α–induced apoptosis in human neutrophils. Human neutrophils (5 × 10^6/mL) were preincubated with antihuman TNFR55 or TNFR75 MoAbs for 30 minutes at 37°C before a 6-hour incubation in the presence or absence of 12.5 ng/mL TNF-α. The extent of apoptosis was then assessed by morphological examination of cytocentrifuge preparations. Data represent mean ± SEM of three separate experiments each performed in triplicate. Where not shown, SEM values are less than 5% of means.

Complementary experiments were performed using the TNFR55-selective TNF-α agonistic mutants E146K and R32W-S86T and the TNFR75-selective mutant D143F. The former peptides have a 3,300- and 5,000-fold lower affinity for the TNFR75 receptor with only marginal (2- and 2.2-fold) reduction in TNFR55 affinity whereas the TNFR75 ligand D143F displays a 30-fold reduced affinity to TNFR75 and no binding to TNFR55.[25,26] Figure 8 shows the ability of both TNFR55 mutants to induce morphological and DNA ladder evidence of neutrophil apoptosis, albeit with a lower potency than recombinant human TNF-α (EC_{50} values, ng/mL: wild-type TNF-α, 5.0; E146K, 82.7; R32W-S86T, 69.5; both $P < .01$ compared with TNF-α). The induction of apoptosis by E146K and R32W-S86T was also inhibited completely by preincubation with TNFR55 MoAb (Fig 9). The TNFR75 selective agonist D143F, which causes a prompt induction of GM-CSF production in TNFR75 transfected PC12 cells that lack TNFR55 expression,[30] did not induce apoptosis even at a concentration of 100 ng/mL (Fig 8). These data imply that TNFR75 facilitates the ability of TNFR55 to induce apoptosis in human neutrophils.

DISCUSSION

We have shown that TNF-α has a unique and bimodal effect on the rate of apoptosis in human neutrophils and have



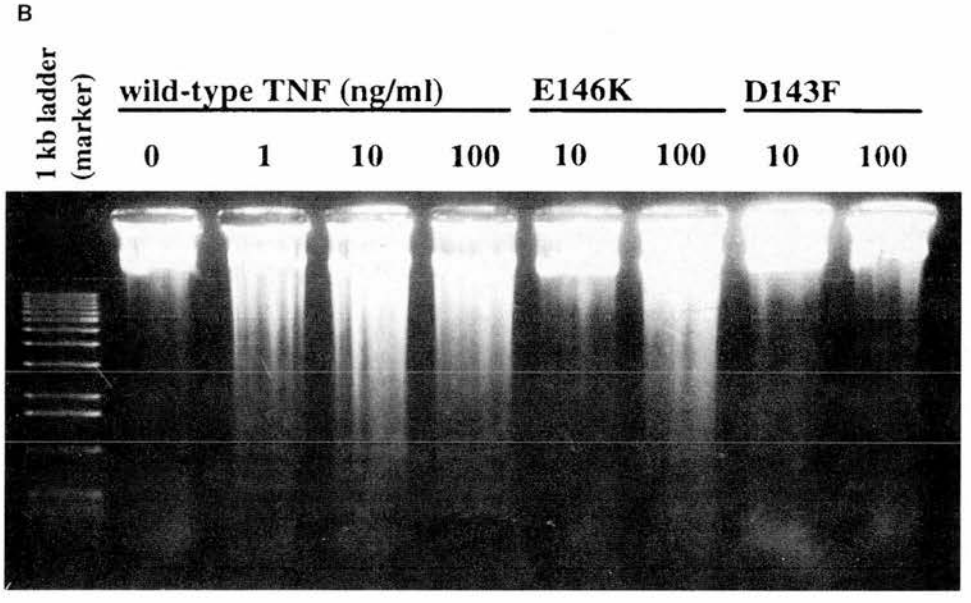


Fig 8. Effect of TNF-α receptor selective mutants on human neutrophil apoptosis. (A) Human neutrophils were incubated with 0.01 to 100 ng/mL of wild-type TNF-α (wtTNF-α, ■), the TNFR55-selective mutants R32W-S86T (●) or E146K (♦), and the TNFR75 selective mutant D143F (▲). Apoptosis was assessed morphologically after 3 hours. Data represent mean ± SEM of three experiments each performed in triplicate. (B) Neutrophils were incubated under identical conditions with 0 to 100 ng/mL wild-type TNF-α, 10 to 100 ng/mL E146K, or 10 to 100 ng/mL D143F. Thereafter the cells were pelletted and incubated overnight in sodium acetate/EDTA/proteinase K/1% SDS before phenol/chloroform DNA extraction as detailed in Materials and Methods. After precipitation with ethanol, 10 μg of DNA was loaded per lane and separated on a 1.2% agarose gel.

provided an explanation for the previous divergent reports that TNF-α either inhibits,[21] has no effect on,[39] or induces apoptosis in neutrophils.[40,41] Our data also reveal that the early proapoptotic effect of TNF-α, in contrast to its priming effect observed in neutrophils in suspension, or indeed the apoptotic effect of TNF-α observed in nonhematopoietic cells, is critically dependent on engagement of both TNFR55 and TNFR75 receptors.

Neutrophils undergo constitutive apoptosis when aged in vitro,[1-7] yet appear highly resistant to any attempt to accelerate this process using physiological agonists. Indeed, we have shown that even stimuli that are potent inducers of apoptosis in other cell types (eg, corticosteroids, elevation of intracellular Ca^{2+} and hypoxia) all inhibit rather than augment the rate of apoptosis in these cells.[17,42,43] The only other physiological agonist reported to induce neutrophil apoptosis is IL-6[44]; however, this effect was small, has not been confirmed by at least two other groups,[40,45] and the extent of apoptosis recorded (10% compared to 15% in the presence of IL-6 at 24 hours) is very different than that reported in all other comparable studies. Hence, TNF-α appears to have the unique ability not shared by other neutrophil priming and activating agents to induce neutrophil apoptosis.

The effectiveness of the TNFR55-selective mutants E146K and R32W-S86T to induce apoptosis in human neutrophils implies that TNFR75 functions to facilitate a death signal primarily initiated via TNFR55. This conclusion is supported by experiments using rat neutrophils where induc-

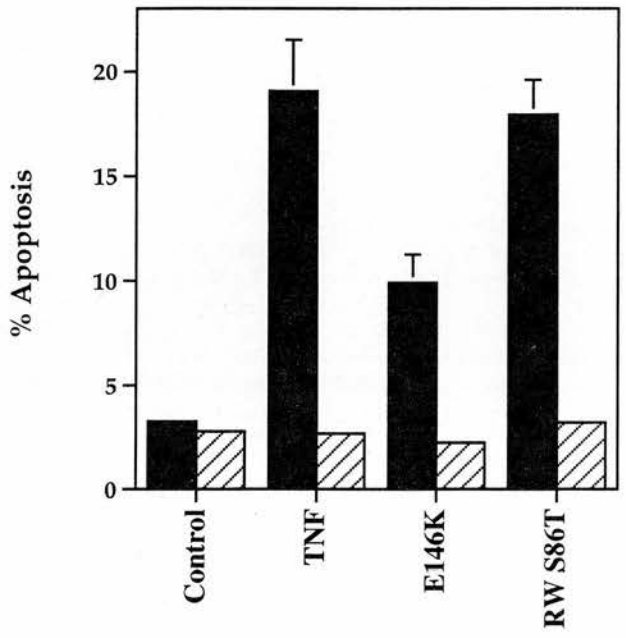


Fig 9. Effect of TNFR55 blocking antibodies on TNFR55-selective mutant-induced neutrophil apoptosis. Human neutrophils (5 × 10^6/mL) were preincubated in the presence (▨) or absence (■) of antihuman TNFR55 MoAbs before stimulation with medium alone (control), 12.5 ng/mL TNF-α, or 100 ng/mL of the TNFR55 selective mutant proteins E146K or RWS86T for 6 hours. The extent of apoptosis was assessed morphologically. Data represent mean ± SEM of three separate experiments, each performed in triplicate.

tion of apoptosis occurs with human TNF-α that does not interact with the mouse TNFR75 receptor.[41] Involvement of TNFR75 in TNF-α–induced death appears to be restricted to hematopoietic cells and was initially thought to reflect the ability of TNFR75 to present TNF-α to TNFR55 via a "ligand passing" effect.[46] However data in PC60 T-cell hybridoma cells, where coexpression of high levels of TNFR75 in addition to low levels of TNFR55 is likewise required to observe TNF-α–stimulated apoptosis, have excluded such a mechanism.[47] The involvement of TNFR75-derived intracellular signals in mediating the death effect of TNF-α is supported by the recent demonstration that TNF-α can induce T-cell receptor-induced apoptosis in $p55^{-/-}$ mice, indicating that under certain circumstances TNFR75 can function alone to induce apoptotic cell death,[18] and similar conclusions have been reached in HeLa cells using TNFR75 selective antibodies.[48] The precise mechanism whereby TNFR75 initiates apoptosis or permits TNFR55-induced death remains uncertain, not least because this receptor subtype has a relatively short cytoplasmic domain with no intrinsic kinase activity or death domain sequence (as found in TNFR55 and Fas).[23] However, the discovery of a TNFR75-associated kinase that phosphorylates both TNF-α receptors provides a potential mechanism whereby TNFR75 could facilitate TNFR55 intracellularly.[49]

The observation that only a proportion of neutrophils undergo apoptosis in response to TNF-α is of particular interest, not least because there was no detectable decline in TNF-α levels over the 20-hour incubation period studied. There is no evidence to support heterogeneous TNF-α receptor expression in neutrophils because TNF-α stimulates uniform CD18-dependent latex bead binding in nearly 100% of neutrophils[50] and flow cytometric analysis demonstrates a single population of TNFR55 and TNFR75 positive cells (Fig 6A). However, heterogeneous neutrophil responses to other stimuli such as LPS augmentation of fMLP-induced Ca^{2+} transients are well documented[51] and other factors such as cell maturity may be important in dictating the susceptibility of individual cells to apoptosis. Our data have also shown a discrete TNFR55-mediated survival effect of TNF-α which is evident at later incubation times and is particularly obvious when the early proapoptotic effect is abolished (Fig 5A); this effect is likely to limit the extent of apoptosis occurring at earlier times. Furthermore, TNF-α induces substantial TNFR55 and TNFR75 loss under our assay conditions (S.A.D., J.M., and I.D., unpublished observations), which again may further curtail the proapoptotic effect of TNF-α.[52] Such a proposal would agree with the findings of Tsuchida et al,[41] who have shown a correlation in rat neutrophils between TNF-α receptor numbers and TNF-α–induced apoptosis.

The loss of the proapoptotic effect of TNF-α when cells were cultured for 6 hours before cytokine addition may well reflect the major decrease in TNFR55 and TNFR75 expression that occurs over this period (Fig 6B and C), but this mechanism does not appear to account for the similar effect observed with PAF where no change in TNFR55 or TNFR75 expression was observed. Although a previous study has reported that PAF induces a 50% decline in total TNFR expression using ^{125}I-TNF-α binding, this effect was obtained after a 2-hour rather than a 5-minute incubation period and used PAF at a concentration of 100 μmol/L.[33] However, it is possible that PAF influences receptor coupling rather than expression and/or that TNF-α is sensitive to PAF-induced protease release. TNF-α has also been shown to enhance thrombospondin-mediated phagocytosis of apoptotic neutrophils by monocyte-derived macrophages,[53] suggesting a potentially synergistic or dual mechanism whereby TNF-α may enhance the removal of neutrophils from an inflamed site by accelerating apoptosis and facilitating clearance.

These data indicate that in addition to its ability to upregulate many aspects of neutrophil function,[54,55] TNF-α also has the capacity, unique among other priming/activating agents, to promote neutrophil apoptosis. Although the neutrophil is a short-lived cell, it is interesting to speculate that depending on the balance of inflammatory mediators present at an inflamed site, there is clearly the potential for neutrophil function and longevity to be either upregulated or downregulated. This effect of TNF-α may explain at least in part certain in vivo findings; for example, how the administration of anti–TNF-α antibodies results in delayed resolution of the neutrophilic alveolitis induced by *Legionella pneumophila*,[56] and why pneumococcus (one of the most potent inducers of alveolar macrophage TNF-α production[57]) causes a form of pneumonia that usually resolves with minimal residual organ damage.[58] In view of recent observations that genetic variations in the TNF-α gene promotor region are associated with differences in constitutive and inducible concentrations of

TNF-α and that this alters the clinical course of malaria and tuberculosis infection,[59] it is also tempting to speculate that similar influences may also have a bearing on neutrophil influx and clearance at sites of inflammation. The recognition that neutrophil apoptosis can be stimulated by TNF-α and that the priming and apoptotic effects of this cytokine can be dissociated may help in the design of more rational and/or selective anti-inflammatory therapy.

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REFERENCES

1. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM, Haslett C: Macrophage phagocytosis of ageing neutrophils in inflammation. Programmed cell death in the neutrophil leads to recognition by macrophages. J Clin Invest 83:865, 1989

2. Savill JS, Henson PM, Haslett C: Phagocytosis of aged human neutrophils by macrophages is mediated by a novel "charge sensitive" recognition mechanism. J Clin Invest 84:1518, 1989

3. Haslett C, Savill JS, Meagher L: The neutrophil. Curr Opin Immunol 2:10, 1989

4. Savill JS, Dransfield I, Hogg N, Haslett C: Macrophage recognition of 'senescent self'; the vitronectin receptor mediates phagocytosis of cells undergoing apoptosis. Nature 342:170, 1990

5. Savill JS, Hogg N, Haslett C: Thrombospondin co-operates with CD36 and the vitronectin receptor in macrophage recognition of aged neutrophils. J Clin Invest 90:1513, 1992

6. Haslett C, Savill JS, Whyte MKB, Stern M, Dransfield I, Meagher LC: Granulocyte apoptosis and the control of inflammation. Phil Trans R Soc London B 345:327, 1994

7. Whyte MKB, Meagher LC, MacDermot J, Haslett C: Impairment of function in ageing neutrophils is associated with apoptosis. J Immunol 150:5123, 1993

8. Meagher LC, Savill JS, Baker A, Fuller R, Haslett C: Phagocytosis of apoptotic neutrophils does not induce macrophage release of thromboxane B_2. J Leukoc Biol 52:269, 1992

9. Cox G, Crossley J, Xing Z: Macrophage engulfment of apoptotic neutrophils contributes to the resolution of acute pulmonary inflammation in vivo. Am J Respir Cell Mol Biol 12:232, 1995

10. Grigg JM, Savill JS, Sarraf C, Haslett C, Silverman M: Neutrophil apoptosis and clearance from neonatal lungs. Lancet 338:720, 1991

11. Savill JS, Smith J, Ren Y, Sarraf C, Abbott F, Rees AJ: Glomerular mesangial cells and inflammatory macrophages ingest neutrophils undergoing apoptosis. Kidney Int 42:924, 1992

12. Begley CG, Lopez AF, Nicola NA: Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in vitro: A rapid and sensitive microassay for colony-stimulating factors. Blood 68:162, 1986

13. Ichinose Y, Hara N, Ohtu M, Aso H, Chikama H, Kawasaki M, Kubota I, Shimizu T, Yagawa K: Recombinant granulocyte colony-stimulating factor and lipopolysaccharide maintain the phenotype of and superoxide anion generation by neutrophils. Infect Immun 58:1647, 1990

14. Brach MA, de Vos S, Gruss H-J, Herrman F: Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. Blood 80:2920, 1992

15. Cox G, Gauldie J, Jordana M: Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. Am J Respir Mol Cell Biol 7:507, 1992

16. Lee A, Whyte MKB, Haslett C: Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. J Leukoc Biol 54:283, 1993

17. Hannah S, Mecklenburgh K, Rahman I, Bellingan GJ, Greening A, Haslett C, Chilvers ER: Hypoxia prolongs neutrophil survival in vitro. FEBS Lett 372:233, 1995

18. Zheng L, Fisher G, Miller RE, Peschon J, Lynch DH, Lenardo MJ: Induction of apoptosis in mature T cells by tumour necrosis factor. Nature 377:348, 1995

19. Obeid LM, Linardic CM, Karolak LA, Hannun YA: Programmed cell death induced by ceramide. Science 259:1769, 1993

20. Mangan DF, Wahl SM: Differential regulation of human monocyte programmed cell death (apoptosis) by chemotactic factors and pro-inflammatory cytokines. J Immunol 147:3408, 1991

21. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A: Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood 80:2012, 1992

22. Genestier L, Bonnefoy-Berard N, Rouault J-P, Flacher M, Revillard J-P: Tumour necrosis factor-α up-regulates Bcl-2 expression and decreases calcium-dependent apoptosis in human B cell lines. Int Immunol 7:533, 1995

23. Tartaglia LA, Ayres TM, Wong GHW, Goeddel DV: A novel domain within the 55kd TNF receptor signals cell death. Cell 74:845, 1993

24. Van Ostade X, Vandenabeele P, Everaerdt B, Loetscher H, Brockhaus M, Lesslauer W, Tavernier J, Brouckaert P, Fiers W: Human TNF mutants with selective activity on the p55 receptor. Nature 361:266, 1993

25. Van Ostade X, Vandenabeele P, Tavernier J, Fiers W: Human tumour necrosis factor mutants with preferential binding to and activity on either the R55 or R75 receptor. Eur J Biochem 220:771, 1994

26. Loetscher H, Stueber D, Banner D, Mackay F, Lesslauer W: Human tumor necrosis factor α (TNFα) mutants with exclusive specificity for the 55-kDa or 75-kDa TNF receptors. J Biol Chem 268:26350, 1993

27. Haslett C, Guthrie LA, Kopaniak MM, Johnstone RB, Henson PM: Modulation of multiple neutrophil function by preparative methods or trace concentrations of bacterial lipopolysaccharide. Am J Pathol 119:101, 1985

28. Nicoletti I, Migliorati G, Oagliacci MC, Grignani F, Riccardi C: A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Meth 139:271, 1991

29. Qu J, Condliffe AM, Lawson M, Plevin RJ, Riemersma RA, Barclay GR, McClelland DB, Chilvers ER: Lack of effect of recombinant platelet-derived growth factor on human neutrophil function. J Immunol 154:4133, 1995

30. Barbara JAJ, Smith WB, Gamble JR, Van Ostade X, Vandenabeele P, Tavernier J, Fiers W, Vadas MA, Lopez AF: Dissociation of TNF-α cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-α mutants. EMBO J 13:843, 1994

31. Van Otteren GM, Standiford TJ, Kunkel SL, Danforth JM, Strieter RM: Alterations of ambient oxygen tension modulate the expression of tumor necrosis factor and macrophage inflammatory protein-1 alpha from murine alveolar macrophages. Am J Respir Cell Mol Biol 13:399, 1995

32. Eggleton P, Penhallow J, Crawford N: Priming action of inositol hexakisphosphate ($InsP_6$) on the stimulated respiratory burst in human neutrophils. Biochim Biophys Acta 1094:309, 1991

33. Schleiffenbaum B, Fehr J: The tumour necrosis factor receptor and human neutrophil function. J Clin Invest 86:184, 1990

34. Lennon SV, Martin SJ, Cotter TG: Dose-dependent induction

of apoptosis in human tumour cell lines by widely diverging stimuli. Cell Prolif 24:203, 1991

35. Xing Z, Kirpalani H, Torry D, Jordana M, Gauldie J: Polymorphonuclear leukocytes as a significant source of tumor necrosis factor-α in endotoxin-challenged lung tissue. Am J Pathol 143:1009, 1993

36. Nathan C, Sanchez E: Tumor necrosis factor and CD11/CD18 (β_2) integrins act synergistically to lower cAMP in human neutrophils. J Cell Biol 111:2171, 1990

37. Abe Y, Osuka Y, Nadata T, Kashu Y, Kimura S: The functional role of 55- and 75-kDa tumour necrosis factor receptors in human polymorphonuclear cells in vitro. Cytokine 7:39, 1995

38. Menegazzi R, Cramer R, Patriarca P, Scheurich P, Dri P: Evidence that tumor necrosis factor-α (TNF)-induced activation of neutrophil respiratory burst on biologic surfaces is mediated by the p55 TNF receptor. Blood 84:287, 1994

39. Kwon MS, Heinz H, Abid H, Leibold W: Differential influences of recombinant TNFα and recombinant IFN-γ on purified polymorpholeukocytes in vitro, in Bonavida B, Gifford GE, Kircher J, Old LJ (eds): Tumour Necrosis Factor/Cachectin and Related Cytokines. Basal, Switzerland, Karger, 1988, p 87

40. Takeda Y, Watanabe H, Yonehara S, Yamashita T, Saito S, Sendo F: Rapid acceleration of neutrophil apoptosis by tumour necrosis factor-α. Int Immunol 5:691, 1993

41. Tsuchida H, Takeda Y, Takei H, Shinzawa H, Takahashi T, Sendo F: In vivo regulation of rat neutrophil apoptosis occurring spontaneously or induced with TNF-α or cyclohexamide. J Immunol 154:2403, 1995

42. Meagher LC, Cousin JM, Seckl JR, Haslett C: Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol 156:4422, 1996

43. Whyte MKB, Meagher LC, Hardwick S, Savill J, Haslett C: Transient elevations of cytosolic free calcium retard subsequent apoptosis in neutrophils in vitro. J Clin Invest 92:446, 1993

44. Afford SC, Pongracz J, Stockley RA, Crocker J, Burnett D: The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. J Biol Chem 267:21612, 1992

45. Biffl WL, Moore EE, Moore FA, Barnett CC: Interleukin-6 suppression of neutrophil apoptosis is neutrophil concentration dependent. J Leukoc Biol 58:582, 1995

46. Tartaglia LA, Pennica D, Goeddel DV: Ligand passing: The 75-kDa tumor necrosis factor (TNF) receptor recruits TNF for signaling by the 55-kDa receptor. J Biol Chem 268:18542, 1993

47. Vandenabeele P, Declercq W, Vanhaesebroeck B, Grooten J, Fiers W: Both TNF receptors are required for TNF-mediated induction of apoptosis in PC60 cells. J Immunol 154:2904, 1995

48. Bigda J, Beletsky I, Brakebusch C, Varfolomeeu Y, Engelmann H, Bigda J, Holfmann H, Wallach D: Dual role of the p75 tumor necrosis factor (TNF) receptor in TNF cytotoxicity. J Exp Med 180:445, 1994

49. Dornay BG, Reddy SAG, Aggarwal BB: Physical and functional association of a serine-threonine protein kinase to the cytoplasmic domain of the p80 form of the human tumor necrosis factor receptor in human histiocytic lymphoma V-937 cells. J Biol Chem 269:19687, 1994

50. Condliffe AM, Chilvers ER, Haslett C, Dransfield I: Priming differentially regulates neutrophil adhesion molecule expression/function. Immunology 89:105, 1996

51. Yee J, Christou NV: Neutrophil priming by lipopolysaccharide involves heterogeneity in calcium-mediated signal transduction. J Immunol 150:1988, 1988

52. Higuchi M, Aggarwal BB: TNF induces internalization of the p60 receptor and shedding of the p80 receptor. J Immunol 152:3550, 1994

53. Ren Y, Savill JS: Proinflammatory cytokines potentiate thrombospondin-mediated phagocytosis of neutrophils undergoing apoptosis. J Immunol 154:2366, 1995

54. Kishimoto TK, Jutila MA, Berg EL, Butcher EC: Neutrophil Mac-1 and MEL-14 adhesion proteins inversely regulated by chemotactic factors. Science 245:1238, 1989

55. Griffin JD, Spertini O, Ernst TJ, Belvin MP, Levine HB, Kanakura Y, Tedder T: Granulocyte-macrophage colony stimulating factor and other cytokines regulate surface expression of the leukocyte adhesion molecule-1 on human neutrophils, monocytes and their precursors. J Immunol 145:576, 1990

56. Skerrett SJ, Bagby GJ, Nelson S: Antibody to tumour necrosis factor impairs pulmonary clearance of Legionella pneumophila. Am J Respir Crit Care Med 149:A871, 1994 (abstr)

57. Simpson SQ, Singh R, Bice DE: Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-α production of murine macrophages. Am J Respir Cell Mol Biol 10:284, 1994

58. Robertson OH, Uhley CG: Changes occurring in the macrophage system of the lungs in pneumococcus lobar pneumonia. J Clin Invest 15:115, 1938

59. Tracey KJ: TNF and Mae West or: Death from too much of a good thing. Lancet 345:75, 1995